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Reconstituting APP and BACE in proteoliposomes to characterize lipid requirements for β-secretase activity

DISSERTATION

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submitted to

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Lucie Kalvodová
born on 26. November 1978 in Prague, Czechoslovakia

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Thesis reviewers:

Prof. Kai Simons, MPI-CBG Dresden

Prof. Bernard Hoflack, TUD Dresden

Prof. Felix Wieland, University of Heidelberg

Based on a true story.

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Abbreviations

2D	two-dimensional
3D	three-dimenstional
Aβ	amyloid-beta-peptide
AD	Alzheimer‘s disease
APP	amyloid precursor protein
BACE	beta-site amyloid protein cleaving enzyme (β-secretase)
cbro	cerebrosides
chol	cholesterol
cmc	critical micellar concentration
DDM	dodecylmaltoside
EM	electron microscopy
FCS	fluorescence correlation spectroscopy
GPL	glycerophospholipids
GSL	glycosphingolipids
GUV	giant unilamellar vesicle
hrs	hours
ld	liquid-disordered phase
lo	liquid-ordered phase
LUV	large unilamellar vesicle
mβCD	methyl-beta-cyclodextrin
min	minutes
OG	octylglucoside
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PL	phospholipids
POPC	palmitoyl-oleoyl phosphatidylcholine
PS	PreScission protease
RT	room temperature
sec	seconds
SEM	standard error of the mean
SL	sphingolipids
TAP	tandem affinity purification tag
TBLE	total brain lipid extract
TEV	tobacco-etch virus
TLC	thin-layer chromatography

1. Summary

Proteolytic processing of the amyloid precursor protein (APP) may lead to the formation of the Aβ peptide, the major constituent of amyloid plaques in Alzheimer's disease. The full-length APP is a substrate for at least 2 different (α and β) proteases ("secretases"). The β-secretase, BACE, cleaves APP in the first step of processing leading to the formation of the neurotoxic Aβ. BACE competes for APP with α-secretase, which cleaves APP within its Aβ sequence, thus precluding Aβ formation. It is thus important to understand how is the access of the α- and β-secretase to APP regulated and how are the individual activities of these secretases modulated. Both these regulatory mechanisms, access to substrate and direct activity modulation, can be determined by the lipid composition of the membrane.

Integral membrane proteins (like APP and BACE), can be viewed as solutes in a two-dimensional liquid membrane, and as such their state, and biological activity, critically depend on the physico-chemical character (fluidity, curvature, surface charge distribution, lateral domain heterogeneity etc.) of the lipid bilayer. These collective membrane properties will influence the activity of embedded membrane proteins. In addition, activity regulation may involve a direct interaction with a specific lipid (cofactor or co-structure function). Interactions of membrane proteins are furthermore affected by lateral domain organization of the membrane.

Previous results had suggested that the regulation of the activity of the α- and β-secretases and of their access to APP is lipid dependent, and involves lipid rafts. Using the baculovirus expression system, we have purified recombinant human full-length APP and BACE to homogeneity, and reconstituted them in large (˜100nm, LUVs) and giant (10-150μm, GUVs) unilamellar vesicles.

Using a soluble peptide substrate mimicking the β-cleavage site of APP, we have examined the involvement of individual lipid species in modulating BACE activity in LUVs of various lipid compositions. We have identified 3 groups of lipids that stimulate proteolytic activity of BACE: 1.cerebrosides, 2.anionic glycerophospholipids, 3. cholesterol.

Furthermore, we have co-reconstituted APP and BACE together in LUVs and demonstrated that BACE cleaves APP at the correct site, generating the β-cleaved ectodomain identical to that from cells. We have developed an assay to quantitatively follow the β-cleavage in proteoliposomes, and we have shown that the rate of cleavage in total brain lipid proteoliposomes is higher than in phosphatidylcholine vesicles.

We have also studied partitioning of APP and BACE in GUVs between liquid ordered (*lo*) and liquid disordered (*ld*) phases. In this system, significant part of the BACE pool (about 20%) partitions into the *lo* phase, and its partitioning into *lo* phase can be further enhanced by cross-linking of membrane components. Only negligible fraction of APP can be found in the *lo* phase. We continue to study the behavior of co-reconstituted APP and BACE in GUVs

The work presented in this thesis has yielded some interesting results and raised further questions. One of the important assignments of this project will in the next stage be the characterization of the impact of membrane domain organization on the β-cleavage. Different domain arrangements that can be hypothesized in cell membranes can be modeled by varying the degree of phase fragmentation in proteoliposomes comprising reconstituted APP and BACE.

2. Introduction

Millions of people worldwide suffer from Alzheimer's disease (AD). The risk of the disease increases with age. Between 6-10% of people aged 65 or below suffer from AD, while almost a half of the population of 85 years or older are affected [1]. AD is a devastating neurodegenerative disease. The disease develops gradually, beginning almost imperceptibly with minor lapses of episodic memory, progressing over the years to result in massive memory and cognition decline, eventually leading to dementia [2]. Invariable physiological changes characteristic for AD pathology include presence of neurofibrillar tangles of hyperphosphorylated Tau protein and the senile plaques composed mainly of the aggregated amyloid β-peptide (Aβ) [3]. While the polymeric Tau structures are intracellular, the Aβ-aggregates accumulate on the cell surface, indicating that the cell membrane should be regarded as a potential participant in plaque deposition process. Indeed, the Aβ peptide is a product of a membrane-associated reaction.

Cellular membranes accommodate a vast variety of membrane associated proteins. Genomic analyses indicate that 25-40% of all proteins are membrane proteins. In this thesis, the focus lies on two such proteins: amyloid precursor protein (APP) and β-site amyloid cleaving enzyme (BACE) that react to produce the aforementioned beta-amyloid (Aβ peptide).

APP structure and function

APP is a relatively large (˜100kDa) integral membrane protein of type I (1 transmembrane segment, large N-terminal luminal ectodomain, short cytoplasmic tail) ubiquitously expressed in various tissues (including the cells of the central nervous system, epithelial cells of various organs, and platelets [4]). The function of APP remains unclear despite the fact that due to its critical role in the development of AD, APP has for a long time been in the

center of attention of a large number of research laboratories worldwide. APP resembles glycosylated surface receptors [5], has been shown to interact with the G_0 subunit of the trimeric G proteins [6], to play a role in cell adhesion [7], and to function in axonal vesicular transport via the interaction of the cytoplasmic domain with the kinesin lightchain [8], gene regulation, cell motility/neuritic outgrowth, and synapse formation [9].

APP localization and trafficking

APP localizes both to the plasma membrane and to intracellular membrane compartments. After synthesis in the endoplasmic reticulum (ER), on its way to the plasma membrane (PM), APP is subjected to further post-translational modifications (N- and O-glycosylation, tyrosine sulfation, and formation of disulfide bridges within its ectodomain). While in neurons, APP is present in two pools, one of the pools present in all membrane compartments of the secretory, endocytic, and lysosomal pathways, and the other highly enriched in axons [10, 11], when expressed in polarized epithelial cells, most of the APP is sorted to the basolateral domain [12]. Thanks to the endosomal targeting motif (YENPTY) within its cytoplasmic tail, APP enters the endocytic pathway via clathrin coated pits [13,12], or by other, dynamin-dependent and putatively raft-mediated routes [14,15]. Like many surface receptors, APP undergoes continued recycling and trafficking [reviewed in 16] throughout its lifetime. The half-life of APP is however relatively short (around 30 minutes) [18,19]. APP is degraded in lysosomes, owing to its GY motif within its cytosolic tail [17]. A phenomenon contributing to the relatively short half-life of APP is its proteolytic processing, including the ectodomain shedding, which is generally an important way of regulating activity of membrane proteins [20,21], and the regulated intramembrane cleavage (RIP) of the remaining membrane-embedded C-terminal stub.

Proteolytic cleavage of APP and endocytosis of the remaining full-length APP together provide for the rapid removal of the cell-surface APP. The estimated half-life for surface-expressed APP is indeed less than ten minutes [22], and thus only minor amounts of the holo-APP (as compared to the total cellular pool) are detected at the plasma membrane [23,24].

APP processing

Enzymes that proteolytically process APP or its fragments are referred to as ″secretases″. Proteolytical processing is necessary for APP to accomplish its physiological functions. Secretion of the APP ectodomain (sometimes referred to as soluble APP, or APPs) is required to fulfill its biological functions in growth regulation and neuroprotection, and, in the case of forms containing the

Kunitz proteinase inhibitor (KPI) domain•, in blood coagulation [25]. Release of the intracellular domain of APP by RIP is essential for it to participate in intracellular signaling and gene regulation♦.

Ectodomain shedding: α- versus β-processing

The full-length APP is a substrate for two major secretase activities. These two competing secretase activities, designated α and β, are responsible for ectodomain shedding of APP, which is a key regulatory step in the generation of the Aβ peptide. After reaching the plasma membrane, a fraction of APP is cleaved by either α- or β-secretases (ectodomain shedding), but the majority remains intact and is rapidly internalized. Another portion of the internalized pool of APP is cleaved (supposedly mainly by β-secretase), and some of the APP is then recycled back to the cell surface, while the rest is delivered to lysosomes for degradation. It follows that regulation of trafficking of APP will have profound effect on its processing. While the α-secretase activity is present mostly at the cell surface, the major location for the β-secretase activity is less clear. It appears that, to a certain extent, the β-cleavage can take place in any of the membrane compartments mentioned above (plasma membrane, endocytic compartments, Golgi/TGN, or perhaps even already in the ER), but the *major site* for β-cleavage cleavage is not known. After the ectodomain had been shed, the remaining membrane-anchored portion of the protein is a substrate for another secretase activity, γ, which is responsible for the intramembrane cleavage.

To make out this seemingly complicated scheme: there are 3 secretase activities that make up 2 possible, competing, mutually exclusive, proteolytical pathways for APP: $\alpha \rightarrow \gamma$, and $\beta \rightarrow \gamma$ (**Figure 1**).

• Several isoforms of APP exist; the 3 major ones are of lengths 695, 750, and 770 residues that arise from alternative splicing. The two longer ones contain the KPI domain, and are common in peripheral tissues. In neurons, the 695 amino acids long form is prevailing.

♦ In fact, a definitive proof for the possibility that the AICD generated by RIP is involved in intracellular signaling and gene regulation is missing.

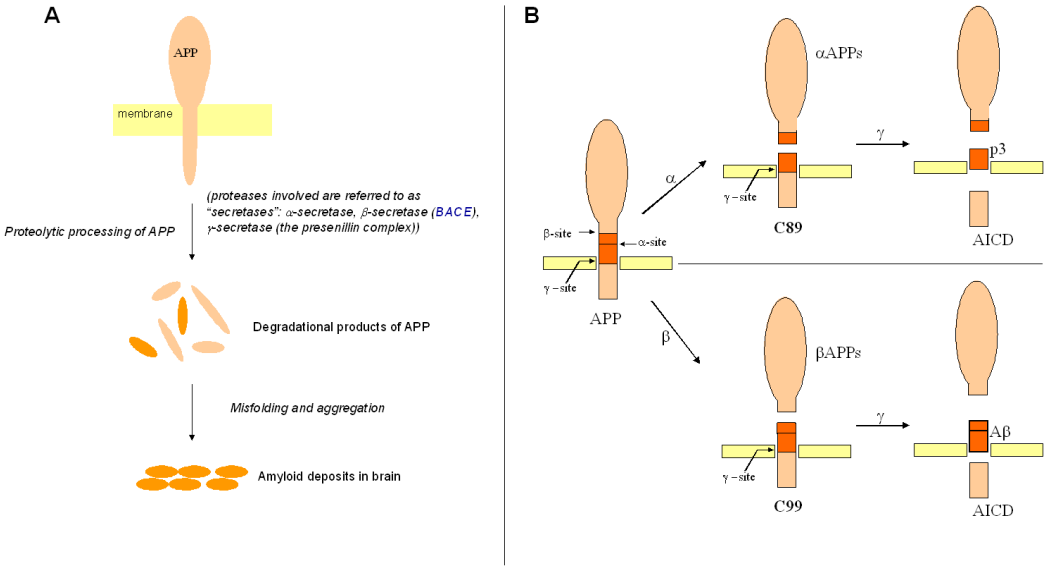

Figure 1 **Proteolytic processing of APP in cells.** *Panel A, APP is a target for at least three scretase activities, that may generate neurotoxic amyloidogenic species. Panel B, The two mutually exclusive proteolytic pathways are depicted. The majority of APP is cleaved by an α-secretase in the middle of the Aβ-sequence, and consequently the γ-cleavage releases the APP intracellular domain (AICD) along with the so-called p3 peptide (non-amyloidogenic). A minor part of the APP pool is cleaved by the β-secretase (BACE), triggering the Aβ generation, which is completed upon the γ-cleavage of the C99 C-terminal fragment.*

α-cleavage is the major cleavage

The major proteolytic pathway, undertaken by ~95% of the APP in neurons, and also a main pathway in non-neuronal cells, is the α - γ pathway, i.e. APP is first cleaved by an α-secretase♦. Importantly, the site of this α-cleavage is located within the Aβ region, in other words, the α-cleavage precludes the formation of the Aβ peptide. Consequently, the remaining membrane-spanning fragment is processed by the γ-secretase complex, leading to the release of the non-toxic p3 peptide.

Proteins with α-secretase activity

It appears that there is more than one protein with α-secretase activity. Several members of the "a disintegrin and metalloprotease" domain family (ADAM, a large protein family of zinc metalloproteases involved in cell-cell interactions and processing of membrane anchored proteins such as TNFα, Notch, Delta and others) have been implicated in α-processing, including ADAM9 and ADAM10. Also TACE (ADAM17) [reviewed in 26], which cleaves TNFα, seems to be in addition involved in regulatory α-processing of APP in fibroblasts [27].

♦ α-secretase ectodomain shedding is analogous to the processing of a series of other integral membrane proteins like the transforming growth factor TGFα, tumor necrosis factor TNFα, the cell adhesion molecule L-selectin, or the growth factor co-receptor syndecan in that the processing consists of a constitutive component and a regulated component that can be activated via protein kinase C (PKC) and by other second messenger cascades.

The β-cleavage leads to the production of Aβ

APP that escapes the α-cleavage gets a chance to enter the alternative proteolytic pathway (the β - γ pathway), which leads to the formation of Aβ. In this case, APP is first cleaved by the β-secretase (BACE, **b**eta-site **a**myloid **c**leaving **e**nzyme[♣], [33]) to allow further processing by the γ-secretase to finally produce the 39 to 43 amino acids long Aβ peptide.

The length heterogeneity of the Aβ peptide arises from the heterogeneity of its C-terminus, i.e. it is owing to the γ-cleavage, and the longer Aβ species are more toxic than the shorter ones owing to their higher hydrophobicity. Thus the factors determining the exact location of the γ-cleavage within the APP sequence are also of great importance in AD pathology.

BACE, the β-secretase: structure

The β-secretase, BACE, is an atypical member of the pepsin family of aspartic proteases; unlike pepsin, gastricsin, or rennin, BACE is an integral membrane protein similar to APP in its topology. It is a ˜60kDa membrane protein of type I, with a relatively bulky ectodomain conferring the active site (two conserved aspartyl protease catalytical motifs (DTGS at residues 93–96 and DSGT at residues 289–292), a single transmembrane domain, and very short cytosolic tail (24 amino acids long). BACE is expressed as a pro-enzyme, with a short pro-domain flanked by a RLPR motif, which is cleaved by furin or other pro-convertases [28, 29]. However, the pro-peptide of BACE does not confer strict zymogen-like properties, but was found to assist in proper folding of the protease domain [30]. BACE is N-glycosylated at 4 sites on its ectodomain [31] and palmitoylated on 3 cysteine residues (1 within the TM segment, 2 within the cytoplasmic tail), the palmitate acyl chains serving as additional membrane anchors [31, 32]. The pattern of disulfide bonding within its catalytical ectodomain is fundamentally different from the disulfide motif of other known aspartic proteases [31], and with respect to the substrate preference and inhibitor profile, BACE has been shown to bear little similarity to other aspartic proteases [34]. This finding, along with the fact that BACE knock-out mice are perfectly viable and healthy [35], implies BACE as a promising and interesting drug target for AD treatment.

BACE, the β-secretase: localization and trafficking

The majority of the cellular BACE pool has been shown to locate to the Golgi/TGN and endosomal compartments [36] - a di-leucine motif (DDISLLK) had been identified within its cytosolic tail [37], and only a fraction of the pool is present at the cell surface. Interestingly, when expressed in polarized epithelial cells, BACE is sorted to the apical domain [38]. This spatial

♣ The β-secretase had been identified by several laboratories almost simultaneously and thus different names were given: BACE, Asp2, memapsin 2

segregation of APP (basolateral) and BACE (apical) was regarded as a paradox, and occurrence of β-cleavage in normal physiology was questioned. It was initially assumed that under normal conditions, it may be solely the α-secretase activity providing for APP ectodomain shedding, and that the β-cleavage of APP may be pathological. Later it was established that both the β-cleavage and the formation of the Aβ peptide are normal physiological events occurring in healthy cells, even though the function of Aβ remains elusive. However, it is not clear whether APP is the main substrate for BACE, since several other proteins had been identified that are cleaved by BACE: the cell adhesion protein P-selectin glycoprotein ligand 1 (PSGL-1) [39], the membrane-bound sialyltransferase ST6Gal I [40], the APP-like-proteins APLP-1 and -2 [41], and supposedly also the low density lipoprotein receptor-related protein (LRP) [42].

Even though it is not the β-cleavage itself that would *per se* lead to the plaque formation, but rather the misfolding and aggregation of the generated Aβ peptide, up-regulation of the β-secretase activity (as well as down-regulation of α-secretase activity) is an issue in amyloidogenesis. Hence it is necessary to establish how is the access of the secretases to APP regulated, and how are the proteolytic activities of the individual secretases modulated.

A simple and efficient way of regulating proteolysis (enzymatic reaction) relies on limited access of the protease (enzyme) to its substrate.

α- versus β-cleavage: how to regulate them?

One obvious way of restricting a contact between two different membrane proteins is by confining them into distinct cellular compartments, and/or possibly dispatching them to separate trafficking routes. To a certain extent, the access of BACE and other secretases to APP is limited in this way (both APP and BACE cycle between the cell surface and intracellular membrane compartments). Since trafficking largely relies on interacting proteins responsible for sorting, modifying (e.g. phosphorylation) and ″packing″ of the cargo, these proteins are also candidates for involvement in β-cleavage regulation [39, 44]. Such proteins that are part of the cellular trafficking machinery interact with sorting motifs within the C-terminal cytosolic tail of APP and BACE. The basolateral sorting signal (YTSI) of APP recruits the microtubule-binding protein PAT-1 [45] and the YENTPY motif mediates interactions with at least three families of adaptor proteins: Fe65, X11, and mDAB1 [39].

The cytosolic domain of BACE contains an acid cluster dileucine motif (ACDL) that binds the VHS (Vps-27, Hrs, and STAM) domain of Golgi-

localized, gamma-ear-containing, Arf-binding proteins (GGA) proteins [46]. GGA proteins were reported to colocalize in the trans-Golgi network and endosomes with BACE and a BACE chimera containing a cytosolic domain of a mannose-6-phosphate receptor. Depleting cellular GGA proteins with RNA interference or mutation of serine 498 to stop the phosphorylation of ACDL resulted in the accumulation of BACE in early endosomes, indicating that GGAs function in BACE recycling from endosomes to TGN and back to the surface.

Although APP and BACE use different trafficking routes, certain fractions reside at any given timepoint within the same membrane compartment. Nevertheless, even within the plane of the same membrane (for example plasma membrane, early endosomal vesicle), a contact between two different membrane proteins (APP and BACE) can be limited by differential partitioning into distinct membrane microdomains, such as lipid rafts [47]. Partitioning of specific lipids and membrane proteins into functional membrane microdomains that may in this way serve as platforms for segregation, sorting, trafficking and localized functioning of membrane components, has become a widely studied subject and appears to be a promising concept underlying numerous processes of cell physiology.

The fluid mosaic model of the plasma membrane – is it **fluid** or **mosaic** in the first place?

In the past few years, the more than 30 years old fluid mosaic model [48] had been repeatedly challenged and put under scrutiny; the key question can be phrased laconically "whether the membrane may be more mosaic than fluid after all" [this question has been phrased before and discussed for example in [49,50]. The efforts aim to extend the fluid mosaic model rather than to reject or contradict it.

The structure of biological membranes has been studied in different contexts for years. While in the 70's and 80's most of the attention had been dedicated to proteins, and lipids were regarded mostly as a fluid medium facilitating protein diffusion, in the past 15 years the interest has been transferred to lipids. (Bio)physicists had realized a long time ago that, regardless of the presence of a huge amount of embedded and associated proteins, biological membranes are, in the first place, lipid bilayers. Thus, different aspects concerning the very essence of lipid bilayers (fluidity, shape/curvature, lateral diffusion of lipids and proteins, phase separation) have been studied from the physical point of view on model systems and *in silico* in detail for years. What these theoretical studies have in common are the typical "bugs" characteristic to most physical studies - (over)simplification (as a rule, membranes were

reduced to phosphatidylcholine membranes, or PC/cholesterol in the best cases, and recently also ternary mixtures have been considered (typically PC/cholesterol/SM)), extremely strict or drastic, ″artificial″, assumptions sometimes bordering on absurdity (equilibrium state, assuming all molecules to be inert and not interacting with each other, overlooking the protein component as insignificant, even though biological membranes contain about 50% of proteins by weight), ignorance of the vertical asymmetry of the membrane, and other. Despite these limitations, model membranes and theoretical modeling eventually started to slowly find their way to integrate into biological concepts that were being formulated based on mostly descriptive, protein-oriented work. The composite picture of membranes as physically characterized, biologically functional environment was (and still is) lacking.

The biological world has been becoming increasingly aware of the results of the physical and theoretical studies, which seemed to have the potential to elucidate some of the poorly understood mechanisms that control localization, multimerization, and assembly of membrane proteins and specific lipids in the plasma membrane, and thus underlie cell-biological events like signaling, vesicular trafficking, protein and lipid sorting. Specialized membrane domains such as coated pits, synapses, and cell-adhesion structures, or caveolae had been long known and described, yet the potential of different membrane lipids to participate in shaping and functioning of such domains remained unrecognized. Such phenomena that biologists observed but could not fully understand demanded introduction of a new concept that would extend the original idea regarding the membrane as a homogenous solvent[*].

Using model membranes, it was found that domains in separate phases, termed liquid-ordered (*lo*) and liquid-disordered (*ld*), can co-exist within the plane of the same membrane[•]. This finding has eventually lead to the realization that

[*] In fact, the original Singer and Nicholson (1972) fluid mosaic model, although emphasizing the fluidity, is compatible with the concept of the fluid bilayer accommodating small (100nm or smaller) subdomains; however, even though studies considering the possibility of existence of small domains in biological membranes periodically appeared, these were generally rather abstract, and biological significance of lipid-driven domain formation was not addressed; interesting papers appeared already in late ‘70 and ‘80 arguing for the existence of domains with different lipid compositions in the plasma membrane of cultured cells [Jain MK, White HB (1977) *Adv Lipid Res* **15**, 1-60, Klausner, RD, Kleinfeld AM, Hoover RL, and Karnovsky MJ (1980) *J Biol Chemistry* **255**, 1286-1295]

[•] The liquid-ordered phase was defined in theoretical work based on five different experimentally determined phase diagrams for DPPC cholesterol [Ipsen, J. H. , Karlstrom, G. , Mouritsen, O. G. , Wennerstrom, H. & Zuckermann, M. J. (1987) *Biochim. Biophys. Acta* **905**, 162-172]. Recently, phase diagram for a more physiologically relevant lipid mixture,

also biological membranes may be laterally compartmentalized independently of the protein component and/or cytoskeleton[#] owing to lipid phase separation and/or preferential interactions of certain lipid species; this idea is the bedrock of the raft hypothesis.

The liquid-ordered phase, the raft phase, requires sterols to form and is characterized by tight lipid acyl chain packing, but rapid lateral diffusion of individual lipid molecules. Lipid rafts are extremely dynamic and at a steady state very small, under the detection limit of microscopy techniques. It is however assumed that rafts can be clustered to form larger and more stable (longer lasting) assemblies.

Rafts had been conceptually included in numerous processes at the cell membranes that require dynamical segregation of proteins or lipids.

Implication of rafts in APP processing

Indeed, rafts had also been implicated in APP processing [51]. Decreased levels of cholesterol and sphingolipids, both of which are indispensable constituents of lipid rafts, correlate with reduced β-cleavage [51], while exogenously added cholesterol seems to decrease α-cleavage [52]; also ceramides, which have been suggested to play a role in subdomain organization and raft coalescence, were proposed as β-cleavage modulators [53]. Besides, mutant BACE linked to a GPI anchor, which enhances its association with detergent resistant membranes[♥] (DRMs), seems to cleave APP more efficiently than the wild type BACE [54].

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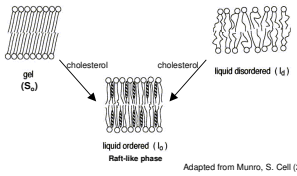
namely POPC/SM/cholesterol, which resembles the composition of DRMs, was published [de Almeida RF, Fedorov A, Prieto M (2003) *Biophys J* **85**, 2406-16]. Looking at this phase diagram (not shown here), one can recognize the function of cholesterol as a fluidity modulator:



Depending on the composition, increasing cholesterol content may either render the bilayer more fluid (for example, moving from single phase (*lo*) composition of 30:10:60 down along the decreasing cholesterol content we arrive at *ld+lo* coexistence region), or more ordered (increasing cholesterol content at the expense of POPC in a mixture of POPC:SM 20:80 which exists in a gel state, we arrive at a *lo+so* coexistence region, for example 10:80:10).

[#] domains dependent on protein components or underlying cytoskeleton had been recognized before (caveolae, coated pits)

[♥] DRMs - detergent resistant membranes; DRMs represent a fraction of membranes (ie lipid membranes and associated proteins) that are insoluble in Triton X-100 at 4°C. DRMs were initially often mistakenly identified with lipid rafts. The discovery of the detergent-insoluble fraction of the membranes was put in context with the work done on model membranes, which revealed that liposomes in the liquid-ordered state are resistant to solubilization with non-ionic detergents [Schroeder R, London E, Brown D. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 12130-34]. Apical domains composed of glycosphingolipids and a specific subset of proteins including the GPI-anchored proteins were hypothesized in polarized epithelial cells

Mechanistically, rafts could be involved in β-secretase activity regulation in at least three ways: 1. ″trapping″ APP/BACE and confining them to the relatively small area of the (short-lived) lipid raft would lead to a drastic increase in local concentration, 2. facilitating delivery of APP/BACE to a cellular compartment where the β-cleavage predominantly happens (supposedly early and recycling endosomes), 3. offering an environment (characterized by its lipid composition different from the bulk rest of the membrane) that presents optimal conditions for cleavage (for example supplying necessary lipid cofactors or co-structures (hypothetical) or providing optimal fluidity).

There is a large body of experimental work done on cells in culture as well as *ex vivo* concerning partitioning of APP and BACE into DRMs [55, 56, 57, 58]. Despite the usefulness of probing DRM association in early stages of characterization of the proteins, and especially for detecting *changes* in interactions of membrane components, this type of experimental approach cannot provide adequate insight into raft connections to APP processing.

When APP and BACE are tested for raft association by antibody cross-linking♠, they copatch with a raft marker PLAP but clearly segregate from a

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[Simons K, and van Meer G. (1988) *Biochemistry* **27**, 6197-6202, and Simons K, and van Meer G. (1988) *J Cell Biol* **36**, 51-58], and when it was later revealed that a Triton X-100 insoluble membrane fraction from mammalian cells was enriched in GPI-anchored proteins, sphingolipids, and cholesterol, it was suggested that this DRM fraction represents an actual subset of membrane domains existing in living cells; various lines of experimental studies eventually converged in the raft hypothesis. It was however soon established that DRMs, defining a fraction of membrane components that behave in a particular way upon a drastic, invasive treatment of the native membrane, cannot be identified with pre-existing domains. Thus it is necessary to emphasize that **DRMs are not rafts**, but that the fact that a protein is found within the DRM fraction merely indicates its increased tendency to associate with liquid ordered phase rather than to dissolve in Triton X-100 micelles, but does not necessarily implicate its partitioning into the *lo* phase (raft phase) in the intact, unperturbed native membranes.

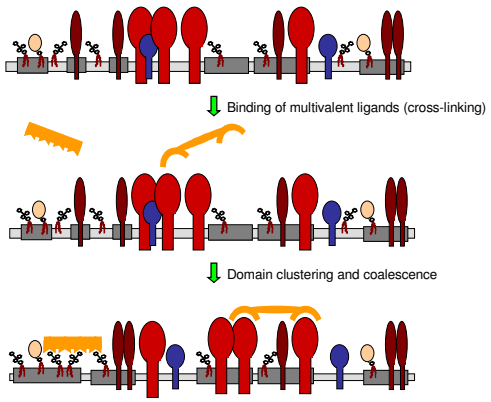
♠ Cross-linking refers to binding of a multivalent ligand specifically recognizing a certain molecule or a group of molecules bearing a common binding motif/moiety (for example glycosylation). Such cross-linking is somewhat similar to multimerization. Cross-linking of raft components is believed to lead to stabilization of raft domains. Cross-linking is schematically depicted in the figure below. When raft components (components strongly associating with the raft phase are depicted in dark red, less raftophilic molecules in bright red; non-raft components are blue; soluble cross-linkers are depicted in bright orange) are ″dragged together″ in this way, they take their surrounding liquid-ordered lipid microdomain (depicted in darker grey) along. Thus, multimerization/cross-linking of raft-associated molecules leads to raft clustering, which can eventually lead to domain coalescence:

non-raft marker (transferrin receptor) and APP and BACE could also be induced to copatch with each other upon cross-linking [51].

These findings indicate that rafts may be involved in regulation of APP cleavage as reaction platforms, but in addition, β-secretase activity is likely to be regulated by direct modulation of the enzymatic activity of BACE. One such regulatory factor is the pH of the surrounding aqueous environment; another mechanism engages membrane lipids, either directly interacting with BACE as cofactors or co-structures, or simply providing optimal bulk membrane properties. We had therefore set about to characterize the lipid requirements for β-cleavage in an artificial, reconstituted system composed of purified proteins and defined lipids. Our data demonstrate that lipids are involved in regulating β-secretase activity of BACE.

Looking at membrane lipid functions in cells

Possibilities to manipulate lipid composition of the cell membranes are limited, and often do not deliver clear-cut results either, since the risk of secondary effects is high. Since rafts are sensitive to cholesterol and sphingolipids levels, manipulating content of these lipids in living cells counts as a useful approach to address raft fuction, but especially cholesterol depletion with methyl-β-



Owing to the fact that cross-linking of raft-associated molecules causes translocation of the whole domain (lipids and associated proteins), cross-linking of one species may lead to co-clustering of multiple raft components. When antibodies against two different raft-associated proteins are simultaneously used for cross-linking, this leads to their concurrent translocation and results in co-clustering, which is observed as strong co-localization in membrane patches. This is referred to as ″patching″ and ″co-patching″[Harder T, Sheiffele P, Verkade P, and Simons K (1998) *J Cell Biol* **141**, 929]. Co-patching is an additional way (besides DRM isolation) to operationally define raft-components. In contrast, cross-linking of non-raft components leads to generation of homogenous assemblies of the cross-linked species.
The concept of cross-linking originates from the observation that extracellularly exposed lipids and membrane proteins can be laterally cross-linked with specific antibodies or multivalent bacterial toxins, causing a redistribution of these plasma membrane elements which tend to form patches on the cell surface [Spiegel, S., S. Kassis, M. Wilchek, and P.H. Fishman (1984) *J Cell Biol* **99,** 1575]. Later, the finding that multimerization of GPI-anchored proteins regulates signalling and affects their segregation in caveolae [Mayor S, Rothberg KG, Maxfield FR. (1994) Science **264,** 1948-51] contributed to experimental interest in using cross-linking as a means of exploring lipid domain organization of the plasma membrane.

cyclodextrin was shown to cause serious side-effects (disruption of the SNARE clusters required for exocytosis [59], block of the clathrin-coated pit formation [60, 61], phosphatidylinositol(4,5)-bisphosphate delocalization from the plasma membrane [62]).

Due to these limitations characteristic for working with living cells, it appears often useful to transfer the studied system to an artificial setup. Membrane proteins can be isolated and purified, and reconstituted in artifical membranes.

Studying influence of membrane lipids on membrane proteins in artificial membrane systems

Reconstituting membrane proteins in artificial bilayer systems (supported membranes or free-standing membranes, like liposomes) offers the advantage of having a full control over the lipid composition of the membrane (actually, over the overall composition of the bilayer; unfortunately we are not yet able to control composition of the two leaflets separately). A number of membrane proteins have been isolated and consequently reconstituted into proteoliposomes in a functional state, including both bacterial and eukaryotic membrane proteins. Such studies provide valuable insights into how membrane lipids influence activity of membrane proteins via directly interacting with them (best known examples include the interaction of GM3 ganglioside with the epidermal growth factor receptor [63],[64], or the chaperone-like function of phosphatidylethanolamine in the assembly of bacterial lactose permease [65]), owing to the collective physico-chemical properties characteristic for the particular lipid composition (typical example of such effect are interactions of peripheral membrane proteins with negatively charged lipid species at the cytoplasmic leaflet of the membrane, or influence of fluidity or bilayer thickness on the activity, for example for (Ca^{2+} + Mg^{2+})-dependent ATPase [66]), or both (more often than not it is difficult to distinguish between the bulk effect and specific effect). However, despite such, however impressive, achievements in the field of membrane protein structure and function[♣] and lipid interactions, approaching membrane protein structure and function in context of lateral domain organization of the membrane has not yet become a challenge for the mainstream research. Essentially all the studies addressing the issue of possible requirements of particular membrane lipids for folding, activity, or trafficking of membrane proteins have been focusing on characterizing these interactions in context of the **entire pool** of the particular protein and the **overall, average lipid composition** of the membrane. Hence the resulting picture will, most likely, fail to reveal the possible existence of

Biomembranes are functionally compartmentalized: Agreed, but the change of view needs to be reflected in experimental design

[♣] the number of solved structures of membrane proteins is increasing [http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html]

different pools of the particular protein, each present within its own membrane environment and thus subject to distinct lipid(or protein)-mediated modulation, independent of the **average,** exceedingly complex, composition of the membrane. In order to unravel the principles and a rationale behind this enormous, puzzling complexity, characteristic for all biomembranes, the aspect of domain-dependent regulation of membrane-associated processes needs to be addressed. Easily said, yet difficult to tackle experimentally. While an enormous number of papers descriptively implicating *lo* domains - rafts - in various physiological processes have been published in last 10 years, the way in which these functional subdomain compartments **mechanistically** affect/facilitate membrane protein interactions in (model) membranes had not yet been **thoroughly experimentally** explored. Only sporadically studies pointing somewhat in this direction emerge [67, 68]. An interesting *in silico* study analyzing the size restrictions posed on microdomains to allow them to deliver an effect on promoting protein-protein interactions was published recently [69].

A promising tool for studying domain-related properties of membrane proteins: GUVs

It is obvious that our toolbox for membrane, and raft, studies has to be updated and expanded in order to match the current needs. One such relatively new, promising tool are the giant unilamellar vesicles that are suitable for studies with reconstituted membrane proteins via employing advanced spectroscopic and microscopic methods [94]. Thanks to the increasing number of studies characterizing phase separation in model lipid mixtures [90,88,93,95], it is possible to achieve micron-scale phase separation in model membranes, and observe these co-existing phases AND the co-existing pools of proteins partitioning into the different phases in GUVs [71].

While GUVs are already becoming a standard tool to study phase separation, membrane curvature effects, and partitioning of membrane proteins, employing GUVs in studies aiming to find a link between phase separation and regulation of activities of associated membrane proteins seems to be untested. Hopefully, work presented in this thesis will contribute to promoting such experimental approaches.

Aim of the project

The general aim of the project underlying this thesis is to characterize how lipids may be involved in modulating β-secretase activity of BACE. Since membrane protein activity may be linked to the lipid composition of the bilayer in different ways, this formulation is rather vague and hard to pursue. Hence individual aspects of lipid-dependent regulation were addressed

separately: First, which membrane lipids influence[♣] the proteolytic activity of BACE, irrespectively of the presence of membrane-anchored full-length APP. Experiments addressing this issue involved comparing specific activity of BACE reconstituted in liposomes of different lipid compositions exerted towards a soluble peptide substrate. Second, partitioning preferences of BACE and APP in systems with co-existing liquid-ordered (*lo*) and liquid-disordered (*ld*) phases were examined; for this purpose, the proteins were reconstituted in giant unilamellar vesicles (GUVs) and their association with *lo* and *ld* phases was probed microscopically and by fluorescence correlation spectroscopy. Third and most important, APP and BACE were co-reconstituted together in liposomes to tackle a question analogous to the first one but this time involving the full-length APP as a substrate. The ultimate goal is not only to identify lipids or lipid compositions that influence β-cleavage of APP, but also to uncover the underlying mechanism, and especially to explore the impact of membrane domain organization/phase coexistence on the APP and BACE interaction.

[♣] already in this simple case, without APP as a substrate, two different modes of action can be postulated for lipid influence. First, direct interaction of a specific lipid in the bilayer or within a microdomain with a distinct lipid composition providing locally higher concentrations of particular lipids; a specific lipid may bind/interact with BACE and thus stimulate/diminish its activity. Second, lipid compostition providing for average ″bulk″ properties of the membrane or of the membrane microdomain like fluidity, curavature, hydrophobic thickness, surface charge and other, which in their end-effect influence BACE conformation and thus activity.

3. Materials and methods

3.1. Materials

3.1.1. Reagents and chemicals

Lipids

Lipids were purchased from *Avanti Polar Lipids* as chloroform solutions or in powder form, and were used without further purification. Gangliosides, PC, PE, PS, and cerebrosides were from porcine brain, while PA was from egg PC, and PI from bovine liver. POPC and DOPC were synthetic.

Detergents

Triton X-100 was from *Fluka*; $C_{12}E_8$, decylmaltoside (DM), dodecylmaltoside (DDM), octylglucoside (OG) and HEGA10 detergents were acquired from *Anatrace*, cholate, deoxycholate, and SDS from *Sigma*.

Resins for affinity purification

Both IgG Sepharose 6 Fast Flow and Glutathion Sepharose beads were purchased from *Amersham Biosciences*.

Fluorescent reagents

The amino-reactive dye CY5 was purchased from *Amersham Biosciences.* DiO fluorescent lipid analog (3,3'-dioctadecyloxacarbocyanine perchlorate ('DiO'; $DiOC_{18}$), Alexa488-cholera toxin subunit B conjugate, and amino-reactive Alexa488 dye were all from *Molecular Probes*.

Antibodies

Rabbit polyclonal antibody 7523 against BACE ectodomain was a kind gift from Christian Haass. Mouse monoclonal antibody 6E10 against aa 1-5 of Aβ was purchased from *Signet,* and β-cleaved-ectodomain-specific antibody ANGU was a gift from Patrick Keller. 22C11 mouse monoclonal antibody against reduced denatured APP was from *Chemicon*. The detection antibodies used for the MSD assay are proprietary to *Meso Scale Discovery (Meso Scale Diagnostics, LLC)*.

Other reagents and materials

OptiPrep density gradient media was from *Axis-Shield* (Oslo, Norway). Cell-culture media and the NuPAGE 4-12% BisTris gels were from *Invitrogen*. All other chemicals were from *Sigma* and *Merck*. The recombinant human BACE ectodomain was from *Oncogene*, and the fluorogenic soluble peptide substrate for BACE (FS-1, Arg-Glu(EDANS)-(Asn670,Leu671)-Amyloid β/A4 Protein Precursor 770 (668-675)-Lys(DABCYL)-Arg) was purchased from *Bachem*.

3.1.2. Cells, constructs, and viruses

The *Spodoptera frugiperda* suspension cell line SF+ was from *Protein Sciences*.

All constructs and viruses were derived from the pFastBac vector, which is a part of the baculovirus expression system supplied by *Invitrogen*.

3.1.3. Equipment

Centrifuges and rotors

Beckman Optima Max (*Beckman Coulter*) tabletop centrifuge was used with rotors TLA45 and TLS55.

Beckman XL-100k Ultracentrifuge (*Beckman Coulter*) was used with Type 45Ti and SW60 rotors.

Other equipment

Miniextruder used for LUV preparation was from *Avanti Polar Lipids*, and protein electrophoresis apparatus X-cell Sure Lock from *Invitrogen*.

Fluorescence-based assays in multiwell format were performed using Ascent Fluoroskan CF platereader.

Confocal fluorescence microscopy and FCS were performed on a commercial ConfoCor2 (*Zeiss*, Jena, Germany) system.

For the MSD assay, Sector Image Reader 6000 along with Discovery Workbench software was used (*MSD Discovery*).

3.2. Methods

3.2.1. Constructs, primers, and baculovirus generation

Human wt BACE1a was amplified by PCR with primers introducing NcoI (containing initial ATG) site at the 5‘ (CGTAGGCCATGGCCCAAGCCCTGCCCTGGCTC) end and XhoI at the 3‘ end (GGAATTCTTAGCTCGAGCCCTTAAGCAGGGAGATGTCATCAGC).

Human wt APP695 was amplified by PCR with primers introducing NcoI (containing initial ATG) site at the 5‘ (CGTAGGCCATGGGTCTGCCCGGTTTGGCACTG) end and XhoI at the 3‘ end (GCTCTAGATTAGCTCGAGCCGTTCTGCATCTGCTCAAAGAACTTGTA).

In constructs used for purification of APP and BACE, APP/BACE were fused to a C-terminal TAP tag via a protease-resistant linker of the sequence (SSGPSGS) followed by the PreScission protease cleavage site (LEVLFQ*GP). The overall modular structure of the construct was thus (APP or BACE)-linker-PreScission-TAP, which was cloned into the pFastBac vector (*Invitrogen*) under the control of the polyhedrin promoter.

The recombinant baculovirus was generated according to the manufacturer‘s instructions.

3.2.2. BACE expression and purification

All buffers used throughout the procedure were based on 50mM HEPES pH 7.25, 150mM NaCl (HBS).

The SF+ (*Protein Sciences*) cells were grown in suspension in a serum-free medium (SF900 II SFM) at 27°C, and were infected with the recombinant baculovirus at the cell density of $1.5x10^6$ cells/mL. The virus stock was roughly titrated by expression levels as judged from western blotting. Cells were

collected by centrifugation (30 min, 400g) 48 hours post infection, and frozen and stored at -80°C.

APP and BACE were purified from isolated membranes of the collected cells. Cells were homogenized in presence of protease inhibitors (chymostatin 6 μg/ml, leupeptin 0.5μg/ml, antipain 10μg/ml, aprotinin 2μl/ml, pepstatin 0.7 μg/ml, APMSF 10μg/ml, and E64 0.1mM) in HBS buffer containing 0.25M sucrose in a hand-held glass homogeniser (20-30 strokes). The homogenate was centrifuged at ˜100,000g for 45min at 4°C. The supernatant was removed, and the resulting pellet was resuspended with the help of the glass homogeniser in HBS + 1% w/v dodecylmaltoside, supplemented with protease inhibitors. The lysate was incubated at room temperature with stirring for ˜ 30min, followed by centrifugation at ˜100,000g for 45min at 4°C. The resulting supernatant was immediately loaded on an equilibrated IgG Sepharose column (1.5mL beads for 1L cell culture) and washed with 10 column volumes (cv) of the running buffer (RB) (HBS + 5% glycerol, 0.5% w/v Triton X-100, pH 7.6), 1 cv RB + 5mM ATP, 2 cv RB, 2 cv RB 2mM EDTA, and finally 5cv RB, usually with reduced detergent content (as little as 0.05% Triton X-100 is sufficient for BACE, while for elution of APP, 0.5% is required; 0.1% Brij35 proved to be a good alternative though). The flow was then stopped and ˜150μl of the PreScission protease (0.5mg/mL) was added to the beads, mixed, and incubated either 3-4 hours at room temperature or 12-16 hours at 4°C. The tagless protein was then eluted at a concentration of 0.25 - 0.8 mg/mL with RB supplemented with 10-50% glycerol. In order to remove the PreScission protease (GST-tagged), the eluate was incubated with glutathion transferase beads for ˜ 30 minutes, and the beads were removed by centrifugation. Purified BACE proved to be stable, and was stored at 4°C, or mixed with 50% glycerol and stored at -20°C, or frozen in liquid nitrogen for long-term storage. In contrast, purified APP quickly degrades at 4°C and was therefore stored with 50% glycerol in -20°C or in liquid nitrogen in small aliquots.

3.2.3. Preparation of large unilamellar vesicles (LUVs)

Large unilamellar vesicles were prepared from a hydrated suspension of multilamellar vesicles by extrusion. Briefly, lipids were mixed in chloroform in a borosilicate glass test-tube, and the solvent was evaporated under a stream of nitrogen for ˜1 hour. The dry lipid film (which was consequently left under vacuum when the original volume of organic solvent was greater than 200μl)

was then hydrated with the LUV buffer (50mM HEPES, 150mM NaCl, 0.2mM EDTA, pH 7.25), which was preheated to/above of Tm of the lipid mixture. Hydration was carried out at/above the Tm with occasional vortexing for at least 30min, or until the suspension appeared homogenous. The resulting suspension was subjected to 3-4 freeze-thaw cycles, and finally extruded through 100nm pore diameter polycarbonate membrane using the Avanti mini-extruder.

3.2.4. Reconstitution of APP and BACE to form proteoliposomes

Typically, LUVs were diluted to ˜1mg/ml, and the detergent (HEGA10 for BACE, OG for APP and APP/BACE co-reconstitutions) was added to the final concentration corresponding to the ″onset of solubilization″ [70], which was determined by turbidity measurements. Typically, this value would be ˜ 0.26% w/v HEGA10 and 0.6% OG, differing slightly for each lipid mix. The liposomes were then incubated for 10min at RT to allow the detergent to equilibrate between the aqueous phase and the liposomes, and consequently, concentrated BACE (typically 0.35mg/ml in 0.3% Triton X-100 and 50% glycerol) was added at a protein to lipid ratio 1:70 to 1:150 w/w. The Triton X-100 concentration was maintained safely below the cmc (typically below 0.008%). The mixture was then incubated at 32°C with shaking for 30min, and consequently for 5min at RT before loading on a Sephadex G-50 column of appropriate dimensions. The size of the column was determined empirically using liposomes with HEGA detergent, so that good separation of liposomes from HEGA detergent was ensured (as little as 0.01% HEGA can be detected using the BCA reagent (unpublished observation)). The proteoliposomes were collected (turbid fractions), adjusted to 12-20% iodixanol, and under-layered under the 2.5% and 5% iodixanol layers in an ultra-clear tube of an appropriate size depending on the final volume. Gradients in 11x34mm tubes were centrifuged for 1h15min in the Beckman TLS55 rotor at 52krpm at 4°C; alternatively, 11x60mm tubes were centrifuged in the Beckman SW60 rotor for 3h at 42krpm at 4°C. Proteoliposomes would typically band at the 5%/12% interface, or would be dispersed in the 5% layer. Empty liposomes would be mostly on the very top of the gradient.

The band was collected and diluted at least 6-fold with LUV buffer, and the proteoliposomes were pelleted for at least 2h, 38krpm, 4°C in the Beckman TLA45 rotor. The supernatant was carefully removed, and the pellets were

resuspended in LUV buffer and stored on ice for several weeks without detectable change in BACE activity; APP-containing liposomes were snap-frozen in liquid nitrogen for longer storage.

For APP/BACE co-reconstitution, the method was modified in order to speed up the procedure. Buffers of higher pH (8.2) were used, proteoliposomes were pelleted on sucrose cushions at 2°C, and the ready proteoliposomes were snap-frozen in liquid nitrogen in small aliquots immediately.

3.2.5. Analysis of protein incorporation in proteoliposomes

Determination of BACE and lipid content in proteoliposomes

An aliquot of the proteoliposomes was loaded on a NuPAGE BisTris 4-12% gel along with APP/BACE mass standards, and run in MOPS buffer. The concentration was determined by densitometry after silver staining (useful range 15-200ng BACE / band, 4-80ng APP).

If desirable, lipid content was estimated from a TLC plate by comparing with appropriate standards.

Proteinase K treatment of reconstituted proteoliposomes

Three equivalent samples of proteoliposomes containing 450ng BACE in LUV were processed in the same way:

1. ″treated″: proteoliposomes supplied with 10μg of proteinase K
2. ″untreated″: non-digested sample, where proteinase K was omitted, to determine maximum possible recovery of the protein
3. solubilized sample supplied with 0.5% Triton X-100 and 0.45% Tween 20[♣] for determination of maximum digestion

Samples were incubated for 1 hour at 37°C, and the reaction was stopped by adding 5mM PMSF and placed on ice. Samples were pelleted at 42krpm, 20min in TLA45 rotor at 4°C, and supernatants and pellets were analyzed separately by western blotting using the 7523 antibody directed against the BACE ectodomain.

[♣] specific activity of proteinase K in 0.5% Triton X-100 + 0.45% Tween 20 is comparable to detergent-free conditions (Qiagen product information).

Sodium carbonate treatment of proteoliposomes

Integral membrane proteins are not extracted from the membrane when treated with 0.1M Na_2CO_3 [72].

Proteoliposomes were treated with 0.1M Na_2CO_3, pH 11.3, and floated in OptiPrep density gradient (step gradient 40,30,5%) prepared in 0.1M Na_2CO_3.

3.2.6. Cholesterol depletion from proteoliposomes

Proteoliposomes were treated with ˜5mM methyl-β-cyclodextrin for 15min at 30°C with gentle shaking, and the activity assayed upon 5-fold dilution with assay buffer. As a control, BACE in Triton X-100 was treated likewise.

3.2.7. Activity assays

In a 96 well plate, typically 12.5ng and 25ng of BACE in proteoliposomes was adjusted to 30μl with the LUV buffer, and the reaction was started by adding 70μl of the reaction buffer (80mM acetate buffer pH 5 supplemented with 90mM NaCl as an osmotic balancer) premixed with the FS-1 substrate (stock solution ˜250mM in 1.5M HAc). The final concentration of FS-1 was ˜8mM, which was enough to saturate 200ng of BACE in 0.025% Triton X-100, or 40ng of BACE in total brain lipid proteoliposomes.

The activity was determined from the linear region on the reaction progression curve (typically up to 1500s) measured as fluorescence at 485nm, excitation 355nm, 37°C using the Ascent Fluoroskan CF reader. The reaction was followed for 4000s in 40s steps, with gentle shaking in between measurements.

3.2.8. Preparation of GUVs with incorporated APP and/or BACE (˝proteo-GUVs˝)

BACE and APP were labeled with the amino-reacitve (maleimide) dye Cy5 directly in elution buffer, and the unreacted dye was separated on a Sephadex G-50 column. The efficiency of labeling was ˜12 Cy5 fluorophors/APP molecule, and ˜2 Cy5 fluorophors/BACE molecule, and specific activity remained unchanged. The labeled proteins were reconstituted in LUVs (when required, the LUVs were prepared with 0.05 - 0.1 mol % DiO) following the same procedure as with non-labeled proteins, except for that the reconstitution buffer was of a 10-fold lower ionic strength (5mM HEPES, 15mM NaCl). After flotation in an OptiPrep gradient, proteoliposomes (proteo-LUVs) were pelleted

(100000g, 1h) and resuspended in milli-Q water containing 10mM trehalose to a final lipid concentration of 20 to 50 mM. To form the proteo-GUVs, a recently developed reconstitution technique was used [71]. The vesicle suspension was deposited onto ITO-coated coverslips and put in the vacuum at 4°C overnight. During membrane de-hydration at low temperature, the vesicles underwent fusion and formed large patches of membranes. Liposomes were re-hydrated in water or 10mM phosphate buffer (pH 7.2) upon an alternate electric field in the flow chamber, as described previously for the GUVs preparation. After 3 to 4 h, numerous unilamellar vesicles formed, with a diameter varying between 10 and 150μm. In the case of lipid mixtures of high phase-transition temperature, membranes were first re-hydrated under the electric field at 55°C for 15min and then cooled down for the following 2 to 3 h.

3.2.9. Confocal fluorescence microscopy and Fluorescence Correlation Spectroscopy (FCS)

(courtesy of Nicoletta Kahya)

Confocal fluorescence microscopy and FCS were performed on a commercial ConfoCor2 (Zeiss, Jena, Germany). Confocal images were taken with the laser scanning microscopy (LSM) module. The excitation light of an Ar ion laser at 488 nm and of a HeNe laser at 633 nm was reflected by a dichroic mirror (HFT UV/488/543/633) and focused through a Zeiss C-Apochromat 40x, NA=1.2 water immersion objective onto the sample. The fluorescence emission was recollected by the same objective and split by another dichroic mirror (NFT 635VIS) into two channels. Detection of the fluorescence emission, after passing a 505-530 nm bandpass filter in the first channel and a 560 nm longpass filter in the second channel, was obtained with two photomultipliers (PMTs). Pinholes (60μm) in front of the PMTs ensured the confocal geometry. FCS measurements were performed by epi-illuminating the sample with the 633 nm HeNe laser ($I_{ex} \approx 1.2$ kW/cm^2). The excitation light was reflected by a dichroic mirror (HTF 633) and focused onto the sample by the same objective as for the LSM. The fluorescence emission was recollected back and sent to an avalanche photodiode via a longpass filter (LP560). Out-of-plane fluorescence was reduced by a pinhole (90μm) in front of the detector. The laser focus was positioned on the topside/bottomside of GUVs, by performing an axial (z-) scan through the membrane prior to the FCS recording. The fluorescence temporal signal was recorded and the autocorrelation function $G(\tau)$ was calculated, according to [73]. The apparatus was calibrated by measuring the

known three-dimensional diffusion coefficient of rhodamine in solution. The detection area on the focal plane was approximated to a Gaussian profile and had a radius of $\approx 0.18\mu m$ at $1/e^2$ relative intensity. Data fitting was performed with the Levenberg-Marquardt nonlinear least-squares fit algorithm (ORIGIN, OriginLab, Northampton, MA). The fitting equation made use of a two-dimensional Brownian diffusion model, assuming a Gaussian beam profile:

$$G(\tau) = \frac{\left(\sum_i \langle C_i \rangle \left(\frac{1}{1 + \tau / \tau_{d,i}} \right) \right)}{A_{eff} \left(\sum_i \langle C_i \rangle \right)^2},$$

where $<C_i>$ is the two-dimensional time average concentration of the species *i* in the detection area A_{eff} and $\tau_{d,i}$ is the average residence time of the species *i*. The diffusion coefficient D_i for the species *i* is proportional to $\tau_{d,i}$. For FCS measurements, three independent GUVs preparations were analyzed and, for each of them, data from at least 20 different GUVs were recorded with 100s acquisition time per FCS measurement. When membrane phase separation was visualized with the LSM, the laser focus was always positioned onto one phase only for the FCS experiment.

Molecular partition coefficients in lipid phases (gel-to-l_d or l_o-to-l_d) were measured by fluorescence intensity analysis of confocal images and by measuring the number of molecules in the focal volume by FCS.

3.2.10. Blue native PAGE (BN-PAGE)

BN-PAGE was performed essentially as described in [74] using a 4-16% gel. The migration of the molecular weight markers is anomalous since the marker that was used was a regular commercial unstained SDS-PAGE marker mixture loaded with BN-PAGE loading buffer. The gel was first extensively washed to remove the dye (Coomassie Brilliant Blue G) and then silver stained.

3.2.11. MSD assay for detection of β-cleaved ectodomain of APP

In this thesis, the name ″MSD assay″ refers to multiwell-format immunodetection assay based on the technology introduced by Meso Scale Discovery. The MSD technology is based on MULTI-ARRAY™ technology, a proprietary combination of patterned arrays and electrochemiluminescence

detection. The actual setup is somewhat similar to ELISA, however instead of the traditional enzyme-linked detection, the MSD assay makes use of the unique electrochemiluminescence detection.

The molecule to be detected is captured by an antibody or other tightly binding ligand which had been preadsorbed to the wells of the plate (or to a particular spot in case of plates with patterned wells). The detection procedure is in principal similar to ELISA up to the actual detection step. The wells are washed and incubated with primary and secondary detection antibodies (or other ligands). The secondary detection ligand carries the special ruthenium-containing tag, which upon excitation with electric current generates luminescence. The luminescent signal is detected optically and quantified.

For the detection of the β-cleaved ectodomain of APP, 1 spot of a 4-spot high binding 96 well plate was coated with $0.5\mu l$ of 100 ng/μl 22C11 antibody in 150mM NaCl, 20mM Tris.Cl pH 7.6. Plates were dried at RT for 1 hour, and consequently blocked with 3% BSA in TBS-T (TBS-T 20mM Tris.Cl, 150mM NaCl, 0.05% Tween20, pH 7.6) for at least 1 hour at 4°C. After washing 3x with TBS-T, samples were applied in total volume of $25\mu l$ and binding was allowed to proceed for 1 hour at RT. Consequently, wells were washed 3x with TBS-T, and primary antibody (b116) was added for 1 hour. After a brief washing step (1 or 2x with TBS-T), wells were incubated with STAG-streptavidin for 30min. Plates were read using the Sector Imager 6000 Reader (MSD Discovery) in MSD-T reading buffer following a thorough wash (4x) with TBS-T. All incubation steps were performed with vigorous shaking.

3.2.12. Electron microscopy

Samples were dried on a grid, fixed with 2% paraformaldehyde and quenched with glycine. For labeling, 7523 (C.Haass) antibody directed against the BACE ectodomain or the 6E10 antibody against the Aβ portion of APP were used together with 6 or 12nm-gold conjugated secondary antibodies. After labeling, samples were fixed as before, washed, and stained with uranyl acetate.

4. Results

4.1. Protein expression and purification

4.1.1. Overexpression of APP and BACE

In order to prepare milligram amounts of active human APP and BACE, we employed the baculovirus expression system along with the suspension-grown insect cell line SF+, which is capable of performing post-translational modifications common in mammalian cells (palmitoylation, glycosylation). We tested two different promoters: a late promoter (polyhedrin) and immediate early promoter (IE1). While the expression under the IE1 promoter only yielded protein in detectable quantities (as judged by western blotting) at ~52 hrs p.i., and then the expression was shut down, the polyhedrin promoter driven expression of APP and BACE was observed over a wider time window, between 40 and 74 hrs p.i.(**Figure 2**). Expression levels were generally higher with the polyhedrin promoter-based constructs, and therefore these constructs were used for further work.

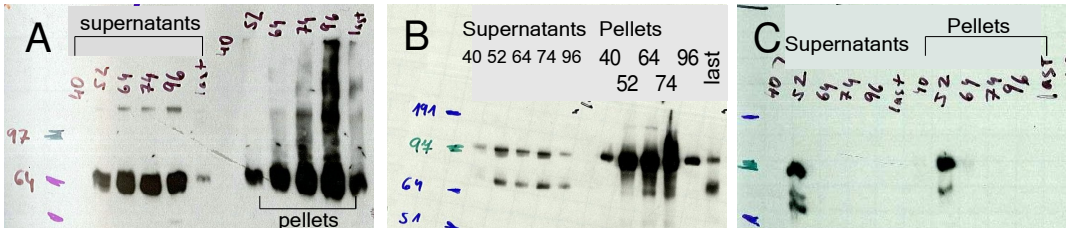


Figure 2 Timecourse of expression of TAP-tagged APP and BACE under the polyhedrin and IE1 promoters.

Panel A Expression of BACE under the control of the late polyhedrin promoter. Cells were collected at the timepoints indicated (the last timepont "last" was collected after 5 days), lysed in 0.1% Triton X-100, and centrifuged to separate soluble material from the insoluble pellet. Expression was first detected at 52 hrs p.i. After 64 hrs, the expression levels did not significantly increase. Panel B Expression of APP under the control of the late polyhedrin promoter. Expression was first detected at 40 hrs p.i. and reached maximal levels around 74 hrs p.i. Panel C Expression of APP under the control of the immediate early promoter1. Expression was detected at 52 hrs p.i. Both APP and BACE were detected using HRP-labelled rabbit-anti-goat antibody (binding to the protein A portion of the TAP tag).

Expression was also observed by immunofluorescence, and efficient plasma-membrane transport was confirmed (**Figure 3**).

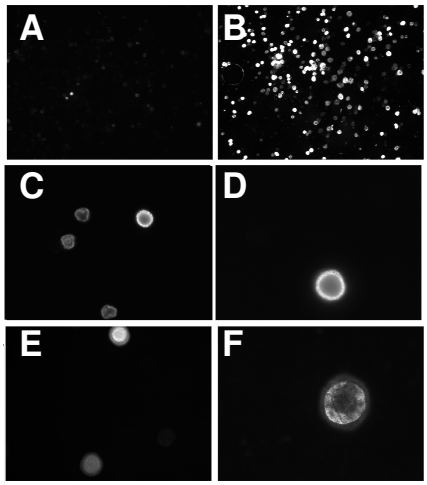


Figure 3 Immunofluorescence on ConA slides, SF+ cells

The cells were attached to concanavalin A coated coverslips and viewed in epifluorescence setup. Panel A - SF+ cells infected with wt baculovirus, stained with 9E10(anti-myc) antibody followed by donkey anti mouse conjug. to CY3 at 10x mag., Panel B - SF+ expressing TAP tagged APP stained with donkey anti mouse CY3 at 10x mag., Panel C - SF+ expressing TAP tagged APP stained with donkey anti mouse CY3 at 40x mag, Panel D - SF+ expressing myc tagged APP stained with 9E10 and donkey anti mouse CY3 at 100x mag, Panel E - SF+ expressing TAP tagged GFP stained with donkey anti mouse CY3 at 40x mag, CY3 channel, Panel F - SF+ expressing TAP tagged GFP stained with donkey anti mouse CY3 at 100x mag, CY3 channel.

4.1.2. Solubilization and purification of APP and BACE

A number of mild, non-denaturing detergents was tested for efficiency of solubilization (Figure 4). These detergents can be ranked in decreasing order of efficiency for APP:

DDM > DM ~ OG ~ Triton X-100~ NP40 ~ $C_{12}E_8$ > Bj35 ~ TW20 ~CHAPS,

and for BACE:

DDM ~ Triton X-100 ~ NP40 ~ $C_{12}E_8$ > Bj35 ~ TW20 ~ DM > OG > CHAPS.

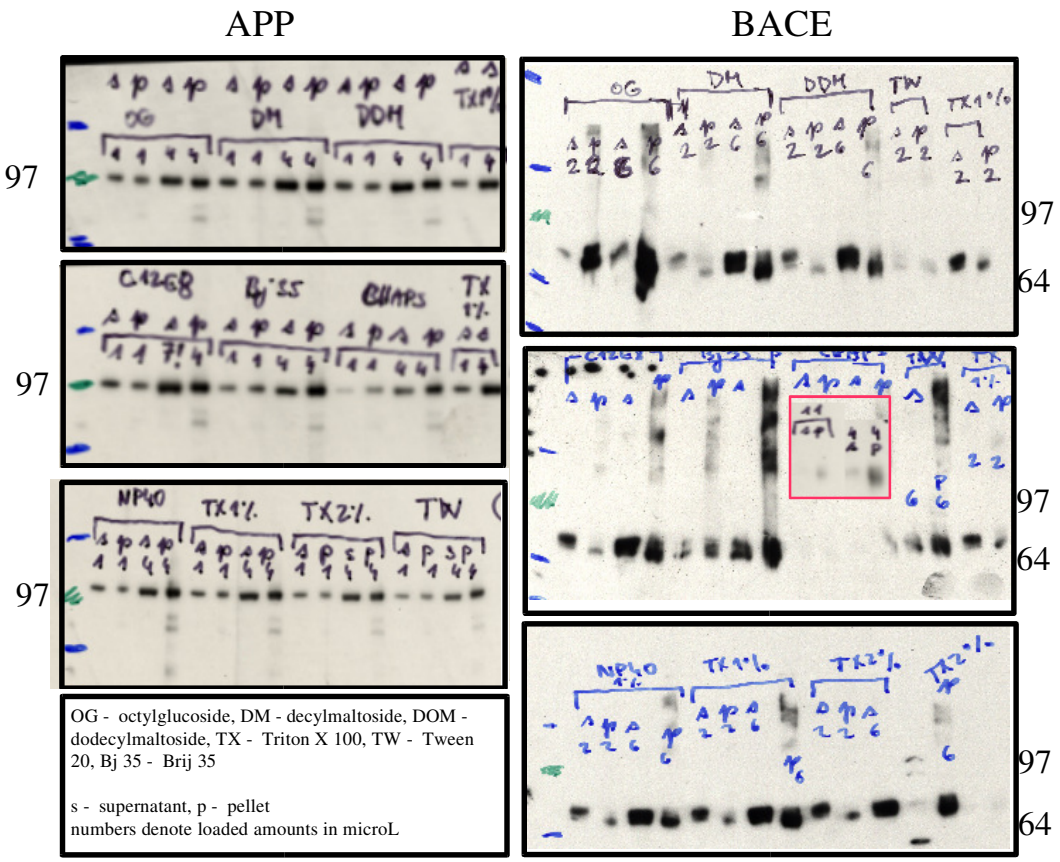
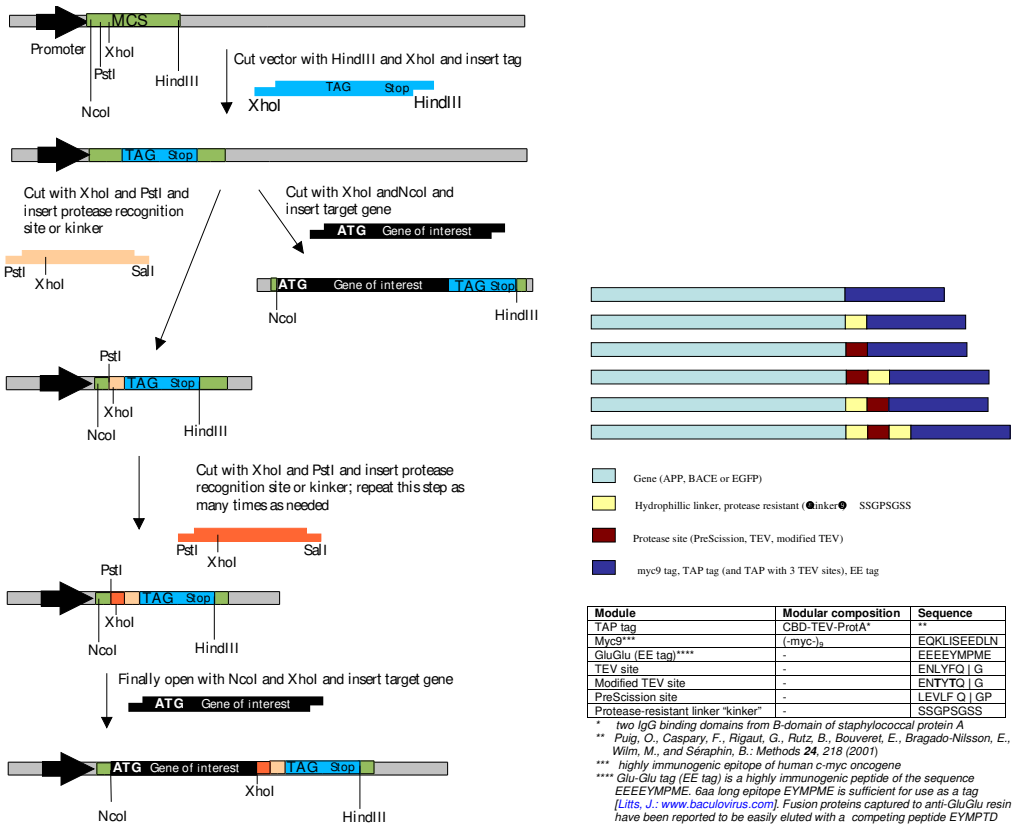


Figure 4 Western blot analysis of the soluble and insoluble fractions of SF+ cells expressing TAP tagged APP and BACE obtained with different detergents. HRP-conjugated rabbit anti goat antibody was used for detection.

Our strategy was to purify the proteins by means of affinity chromatography, relying on specific proteolytical cleavage of the tag to facilitate highly specific elution of the tag-free protein. A series of similar constructs comprising a cleavable affinity tag was used to generate viruses and evaluate the efficiency

of cleavage. The constructs differed in the arrangement of the sequence modules: the C-terminal TAP or $(myc)_9$ tag was linked to the protein sequence via a linker comprising one or two short, inert spacers (protease resistant, proline containing "kinker") and either PreScission or TEV protease recognition site. The constructs and their generation is described in detail in Figure 5.



Module	Modular composition	Sequence
TAP tag	CBD-TEV-ProtA*	**
Myc9***	$(myc)_9$	EQKLISEEDLN
GluGlu (EE tag)****	-	EEEEYMPME
TEV site	-	ENLYFQ | G
Modified TEV site	-	ENTYTQ | G
PreScission site	-	LEVLF Q | GP
Protease-resistant linker "kinker"	-	SSGPSGSS

* *two IgG binding domains from B-domain of staphylococcal protein A*
** *Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Séraphin, B.: Methods **24**, 218 (2001)*
*** *highly immunogenic epitope of human c-myc oncogene*
**** *Glu-Glu tag (EE tag) is a highly immunogenic peptide of the sequence EEEEYMPME. 6aa long epitope EYMPME is sufficient for use as a tag [Litts, J.: www.baculovirus.com]. Fusion proteins captured to anti-GluGlu resin have been reported to be easily eluted with a competing peptide EYMPTD*

Figure 5 Generation and resulting modular composition of the constructs.

We have developed modular system for easy construction of C-tagged targets. The modules used were TAP tag (the TAP tag consists of a calmodulin binding domain linked to the C-terminal protein A domains via a TEV site), modified TAP tag (3 chained TEV sites for more efficient cleavage), myc9 tag, EE tag, PreScission site, TEV site and a modified, less hydrophobic TEV site (ENTYTQ — G), and a protease-resistant linker ("kinker") (SSGPSGSS). Targets (APP,BACE, EGFP) were amplified by PCR reaction and inserted to the generated vectors with varying constellation of the modules.

In summary, all constructs performed reasonably well in facilitating protein pick-up and tag removal, however, it appears that it is useful to include a spacer at least upstream of the protease recognition site to preclude any potential sterical occlusion of the site, which would restrict the access of the protease to the site and thus would lead to inefficient cleavage. The results are reviewed in Figure 6. These results may be generally valid for any target protein.

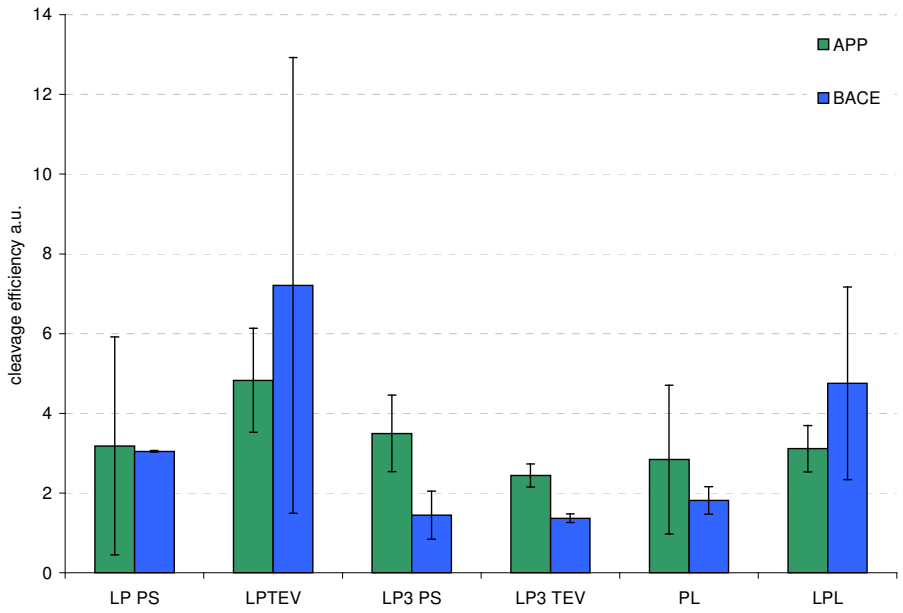


Figure 6 Tag release efficiency in TAP-tagged constructs.

LP PS: linker-PS-TAP, cleaved with PreScission, LPTEV: linker-PS-TAP, cleaved with TEV (ie within the TAP tag), LP3 PS: linker-PS-TAP with 3 TEV sites, cleaved with PreScission, LP3 TEV: linker-PS-TAP with 3 TEV sites, cleaved with TEV, PL: PS-linker-TAP, cleaved with PreScission, LPL: linker-PS-linker-TAP, cleaved with PreScission.

For large-scale purification of APP and BACE, constructs pFB-APP-linker-PS-TAP and pFB-BACE-linker-PS-TAP were used. Single step purification from 1L of 48 h p.i. culture using the IgG Sepharose followed by a simple polishing step on the glutathion resin to remove the GST-tagged PreScission protease yields more than 2mg of homogenous (as judged by silver stained SDS-PAGE) preparation of tagless APP or BACE, eluted at a concentration of 0.1 – 0.8 mg/mL or 0.1 – 0.5 mg/mL respectively **Figure 7**.

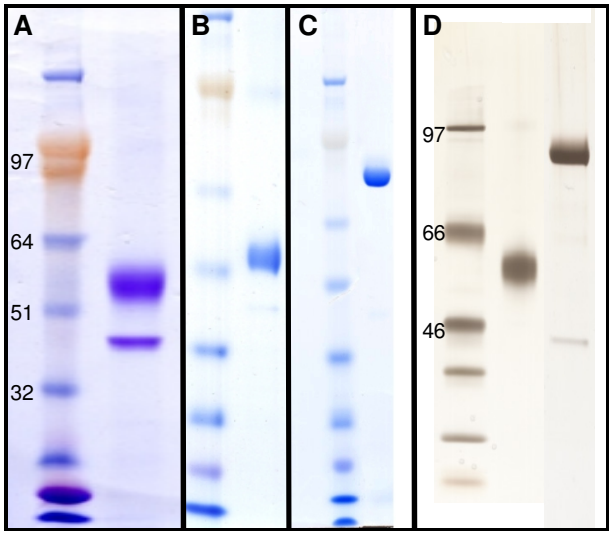


Figure 7 SDS-PAGE analysis of the purified BACE preparation.

A - Coomassie Brilliant Blue R-250 stained gel: First lane – prestained molecular weight marker, second lane – BACE (~2μg); the lower molecular weight band is the GST-tagged PreScission protease used for elution. It is later removed with the help of glutathion sepharose. B – SimplyBlue stained BACE after PreScission removal, C – SimplyBlue stained APP preparation, D - silver stained gels: first lane – molecular weight marker, second lane – BACE (~200ng), third lane APP (~300ng). All gels were 4-12% NuPAGE gel run in MOPS buffer

4.2. Activity of the purified proteins

BACE was capable of processing both a peptide substrate mimicking the cleavage site (**Figure 8**) of the APP protein and the APP protein itself (see further). The purified APP was cleaved by BACE at the correct site (see further).

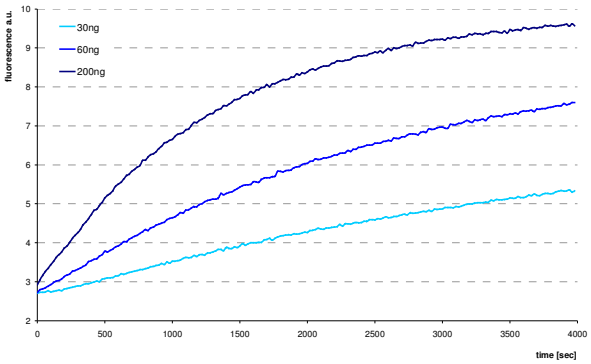


Figure 8 Reaction progress curves recorded for cleavage of the amyloido-mimetic peptide substrate FS-1 by BACE. *Curves resulting from using different amounts of BACE (indicated) are shown. Concentration of the substrate was constant.*

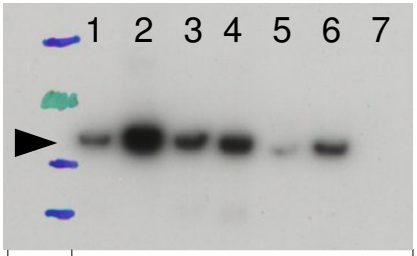
4.2.1. Optimal cleavage conditions *in vitro*

In vitro, optimal pH for cleavage had already been reported for the soluble BACE ectodomain to be 4.5 [1]. Concerning the pH, we have obtained very similar results with the purified full-length BACE (pH optimum ~ 5, data not shown). However, unlike the soluble ectodomain, the full-length transmembrane protein requires a detergent for solubility – and activity. The identity and concentration of detergent is critical. Anionic detergents (cholate, deoxycholate, SDS) were found to be strongly deactivating already below the cmc, and while sugar-based detergents (dodecylmaltoside, octylglucoside) were able to support BACE activity at a particular concentration (OG below or at around 0.5xcmc, dodecylmaltoside up to 1xcmc), OG became strongly deactivating at higher concentrations (no activity of BACE observed at 2xcmc OG). In our assays with solubilized BACE, we have routinely used 0.025% w/v Triton X-100♣.

Since the full-length APP_{695}♠ does not exhibit any easily measurable activity, its "activity" was only assessed in terms of being able to serve as a substrate for BACE. We have found that the purified APP was cleaved by BACE at the correct site (as judged by sequence-specific antibody reactivity; the cleavage site is `EVKM|DA` at the `596|597` position) in Triton X-100, as well as in proteoliposomes (**Figure 9**). Purified APP degrades slowly, and at a lower pH the degradation proceeds faster, which results in non-specific depletion of the β-secretase substrate, and thus the BACE activity is deceptively weaker - the production of the β-cleaved ectodomain is lower. Therefore all the assays involving the full-length APP were performed at pH 6.6 – 6.8.

♣ however, this turned out not to be the best choice, as Triton from different suppliers, as well as different batches of the Triton from the same supplier behaved very differently in terms of supporting BACE activity; an effort was made to use the very same aliquot of the detergent for all experiments, however, this was not always possible. In addition, it appears that the quality of Triton deteriorates over time.

♠ the longer isoforms containing the KPI domain can be assayed for their inhibitory effect on serine proteinases



pH	5	7	5	5	5	7	-
Inh	-	-	+	+	-	-	-

Figure 9 Detection of the β-cleaved ectodomain of APP by SDS-PAGE and Western blotting.

The cleavage was performed at different conditions (pH 5 or 7, presence of inhibitors, cleavage in TX100 or in brain lipid proteoliposomes) and equivalent amounts were loaded. β-cleaved ectodomain was detected with an antibody (ANGU) specific for the newly generated N-terminus of the cleaved ectodomain. The lanes are assigned to the particular conditions below.

Lane 1...brain proteoliposomes, pH 5
Lane 2...brain proteoliposomes, pH 7
Lane 3...brain proteoliposomes, pH 5, in presence of protease inhibitor cocktail
Lane 4...brain proteoliposomes, pH 5, in presence of BACE inhibitor (Inhibitor III, Calbiochem)
Lane 5...Triton X-100, pH 5
Lane 6...Triton X-100, pH 7
Lane 7...50ng full-length APP

4.3. Protein reconstitution in LUVs to form proteoliposomes

The successful method for reconstituting both APP and BACE, separately or together, was reconstituting the proteins into preformed LUVs that had been pre-saturated with detergent. The detergent that gave best results for BACE was HEGA10, while the highest recoveries of liposomes-associated APP were obtained with octylglucoside. Protein insertion was followed by gel filtration by which means virtually all detergent was removed.

4.3.1. Recovery and specific activity of BACE reconstituted in total brain lipid vesicles

The detergent-mediated reconstitution procedure presented here relies on incorporation of protein into preformed liposomes [70] and yields BACE reconstituted directionally (>80% ectodomain outside, **Figure 10**) in large

unilamellar vesicles; the yield was typically 15 – 30% of protein input by weight. The sidedness of the insertion did not depend on the lipid composition of the vesicles. Stable membrane-spanning insertion of BACE was confirmed by treatment with 0.1M Na_2CO_3, pH 11.3 followed by flotation in a density gradient. Virtually all BACE (as judged by silver stained SDS-PAGE) remained associated with the lipids (floating fraction – **Figure 11**). In addition, we performed immunoelectronmicroscopy of the total brain lipid proteoliposomes. We observed vesicles ~ 50 – 200 nm in diameter, heavily labelled with anti-BACE antibody (**Figure 12**). Virtually all immunodetected BACE was found to be membrane associated.

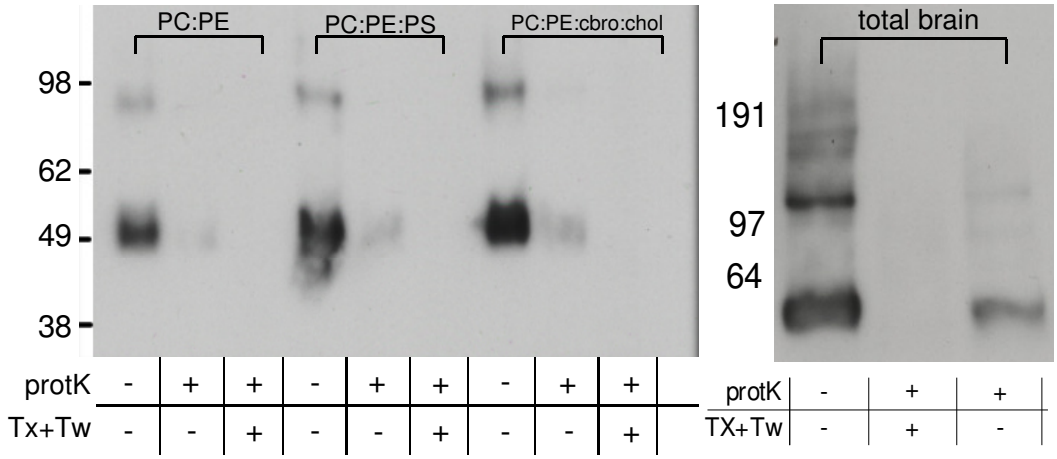


Figure 10 Analysis of the topology of reconstituted BACE by western-blotting.

Proteoliposomes of different lipid compositions were treated with proteinase K either in presence of detergent (TX+Tw) or without detergent, as indicated below the blot. For details, see the Materials and methods *section.*

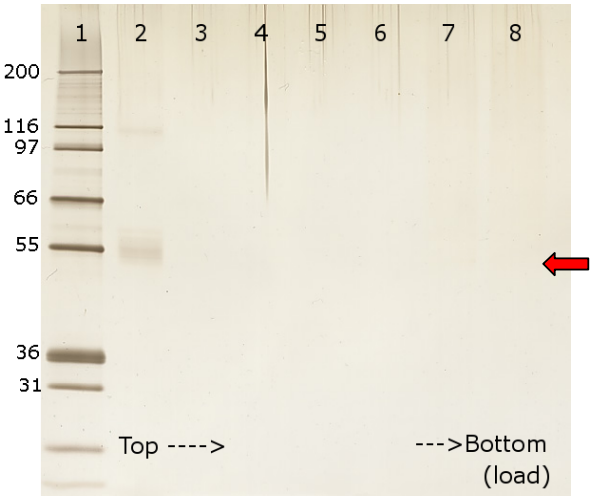


Figure 11 SDS-PAGE analysis of proteoliposomes treated with 0.1M sodium carbonate after flotation in a density gradient.

Lane 1 – molecular weight marker, lanes 2 – 8 fractions from top (floating fraction associated with lipids) to bottom (load).

The specific activity of BACE reconstituted in total brain lipid vesicles was at least 3-fold higher than that of the purified BACE in 0.02% Triton X-100, as judged by cleavage of the soluble fluorogenic substrate which mimics the cleavage site of the Swedish APP mutant.

Proteoliposomes consisting of BACE and total brain lipids were characterized by high reconstitution efficiency, reproducible activity, and stability (activity remains unchanged for several months when stored at 4°C). BACE reconstituted in total brain lipid liposomes was therefore chosen as a standard, and reconstitution in total brain lipid liposomes was always performed as a control together with other samples. In this section, the specific activity of BACE will be expressed as a percentage of the specific activity determined for brain lipid proteoliposomes.

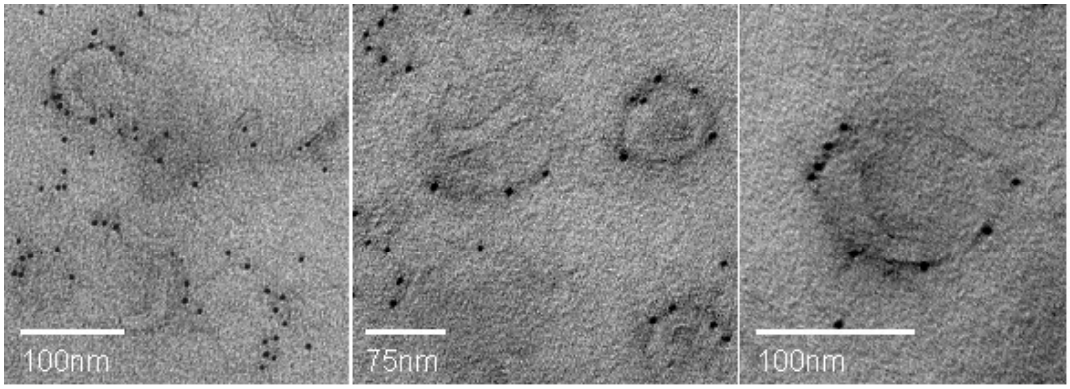


Figure 12 Immunoelectronmicroscopy of the proteo-LUVs probed with anti-BACE antibody recognizing its ectodomain followed by 6nm-gold conjugated secondary antibody.

4.4. BACE activity in proteoliposomes of different lipid compositions

4.4.1. Activity in complex lipid mixtures

There are hundreds of lipid species present in cells, and the reasons for such diversity are poorly understood. One way to study this complexity is to analyze how lipids regulate activities of membrane proteins in a reconstituted system. We therefore dissected the total brain lipid extract, and attempted to elucidate which lipid species are responsible for supporting BACE activity.

As a control, a mixture mimicking the total brain lipid extract was mixed from individual components based on the known glycerophospholipid composition (data from Avanti catalog) and estimated amounts (TLC) of SM, glycosphingolipids and cholesterol. The composition was as follows: PC:PE:SM:cerebrosides:chol:gangliosides:PS:PA:PI (10:17:27:10:15:5:11:3:2 w/w).

BACE activity in this mixture was determined to be 95±12% of TBLE (total brain lipid extract).

Since interpretation of the effects caused by a particular lipid species in complex lipid mixtures such as total brain lipids is problematic, we proceeded to investigate BACE activity in various simpler lipid mixtures of defined headgroup compositions, ranging from simple pseudo-binary mixtures to more complicated mixtures of up to 6 components.

4.4.2. Specific activity of BACE reconstituted in glycerophospholipid and glycerophospholipid:cholesterol vesicles

Glycerophospholipids are the most abundant phospholipids in living cells, of which phosphatidylcholine accounts typically for up to 50% in mammalian cells. Specific activity of BACE in pure PC (brain PC or synthetic POPC) vesicles was not determined due to extremely low recoveries (<5%). It was clear, however, that specific activity in PC vesicles was several-fold (at least 3-5 -fold) lower than that of BACE in TBLE vesicles.

Recoveries improved somewhat when cholesterol was included, therefore POPC:cholesterol 2:1 (mol/mol) was used instead. Specific activity of BACE in POPC:cholesterol vesicles is ~5-fold lower than in TBLE vesicles (**Figure 13**). It has been shown that membrane proteins often require PE for activity, PE serving as a "chaperone" [75] and, along with other "nonbilayer lipids", PE seems to be important for maintaining the physical state of the bilayer, allowing the membrane to readily undergo local rearrangements in response to changes in external conditions [76]. However, in the case of BACE, including 20% of PE did not lead to any significant increase in BACE activity (26±12 % relative to TBLE, **Figure 13**).

On the other hand, there was a strong effect of negatively charged phospholipids on BACE activity. PC:PS (80:20 w/w) supported BACE activity to 58±4 % of TBLE, while mixtures of PC:PE:PS and PC:PE:PA (60:20:20 w/w) brought the specific activity of BACE virtually to the level of TBLE, resulting in 99±9 %, and 135±31 % of TBLE activity, respectively (**Figure 13**).

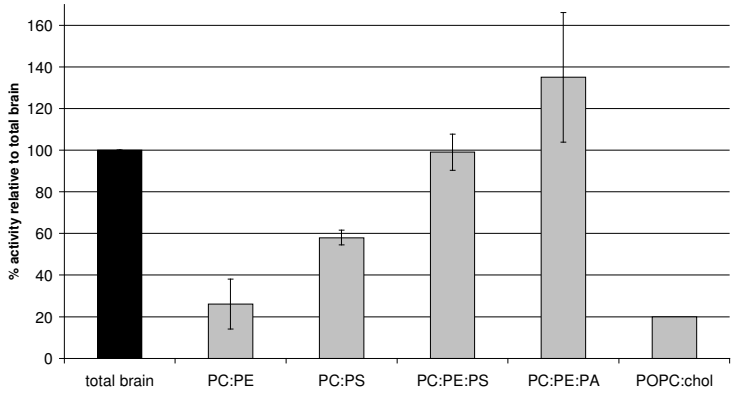


Figure 13 Specific activity of BACE reconstituted in vesicles composed of glycerophospholipids. *PC, PE, and PS were of brain origin except for cholesterol (sheep wool) and PA (produced from egg PC). experiments Where applicable, PE, PS, and PA are each present at 20% w/w. The last bar corresponds to a mixture of POPC:chol 2:1 mol/mol. BACE was reconstituted in LUVs of given compositions by detergent mediated reconstitution followed by flotation in a density gradient to separate resulting proteoliposomes from unreconstituted protein. BACE content was determined in each sample, and specific activity of BACE in each particular mixture measured and is expressed in % relative to the specific activity of BACE in total brain lipid protoeliposomes.*

4.4.3. Specific activity of BACE reconstituted in vesicles composed of glycerophospholipids, (glyco)sphingolipids, and cholesterol

Sphingolipids and cholesterol are essential components of lipid rafts, and various pseudo-ternary mixtures of PC, SM, and cholesterol mixed at ratios that allow for *ld-lo* phase coexistence, have been shown to be useful to imitate lipid rafts. Specific activity of BACE in the *lo* phase forming mixture of PC:SM:chol (1:1:1 or 2:2:1 mol/mol) did not dramatically differ from POPC:chol, resulting in 29±16 % of TBLE activity.

In order to assess the role of cholesterol in modulating membrane properties relevant to BACE activity, we used proteoliposomes consisting of PC:PE:SM:gangliosides supplied with increasing amounts of cholesterol (0, 7.5, and 15% w/w) substituting for SM (25, 17.5, and 10% w/w final) to yield cholesterol:sphingolipid molar ratios of 0, 0.4, 2.5. There was an increase in BACE activity along with increasing cholesterol:sphingolipid molar ratios as follows: 18±1% for PC:PE:SM:gangliosides 50:20:25:5 w/w, 28±1% for PC:PE:SM:gangliosides:**chol** 50:20:17.5:5:**7.5** w/w, and 36±1 % TBLE for PC:PE:SM:gangliosides:**chol** 50:20:10:5:**15** w/w (**Figure 14A**) This last mixture is listed in **Table 1** and is marked ″0″.

When sphingolipids in this mixture (SM and gangliosides) were replaced with cerebrosides (PC:PE:cerebrosides:chol 50:20:15:15 w/w – mixture 1 in **Table 1**), specific activity was determined to be 46±4%. Even though this increase is relatively small, the trend seems to be confirmed in another mixture containing both cerebrosides (12%) and a small amount of SM (5%), which is present at the expense of lowering PC content accordingly (PC:PE:SM:cerebrosides:chol 45:20:5:15:15 w/w – mixture 3), in which BACE displays specific activity of 58±6 %, representing ~20% increase (**Figure 14B**). In addition, we found that cerebrosides, as well as synthetic C8-glucosylceramide, greatly enhance the activity of purified soluble ectodomain of BACE in presence of Triton X-100: ~20nM BACE in presence 0.1mM cerebrosides or C8-glucosylceramide displays almost 9-fold or 4-fold higher activity than a control in Triton X-100 only. Such increase was only observed with cerebrosides and glucosylceramide, and not with any other lipids in solubilized state. However, only a mild effect was observed with the full-length BACE (~1.2 fold increase with 0.1mM glucosylceramide over the control). This may be due to the interference of Triton X-100, since higher concentrations are necessary for full-length BACE.

The effect of cerebrosides was futher investigated in mixtures including anionic phospholipids.

	PC	PE	SM	cbro	chol	PS	PA	**activity**	± SEM
1	44	18	0	12	26	0	0	**46**	4
2	35	18	14	0	26	8	0	**51**	3
3	40	18	5	12	26	0	0	**58**	6
4	26	18	23	0	26	8	0	**61**	5
5	35	18	0	12	26	8	0	**62**	2
6	31	18	5	12	26	8	0	**67**	5
7	35	18	5	12	26	0	5	**78**	4
8	22	18	5	12	26	17	0	**97**	7
9	46	18	5	0	26	0	5	**98**	12
10	34	18	5	0	26	17	0	**64**	10
"0"	44	18	9	0	26	2% ganglios.		**36**	1

Table 1 Results and compositions for lipid mixtures consisting of glycerophospholipids, sphingolipids, and cholesterol. *Composition is given in mol%. All lipids were of brain origin except for cholesterol (sheep wool) and PA (produced from egg PC). BACE was reconstituted in LUVs of given compositions by detergent mediated reconstitution followed by flotation in a density gradient to separate resulting proteoliposomes from unreconstituted protein. BACE content was determined in each sample, and specific activity of BACE in each particular mixture measured. Activity represents specific activity of BACE in % relative to BACE reconstituted in total brain lipid proteoliposomes. The value represents the average of results obtained from 2-7 independent reconstitution experiments.*

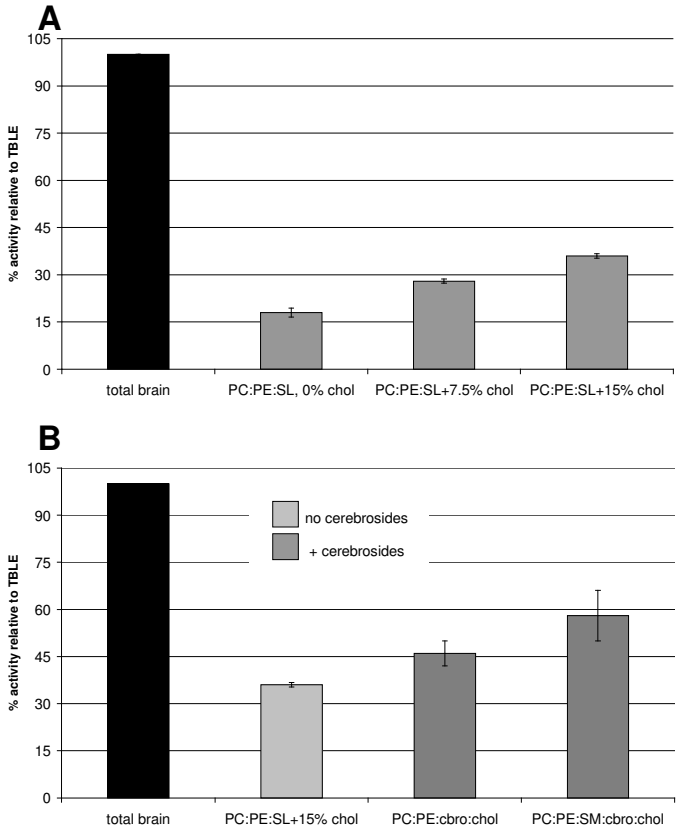


Figure 14 Influence of cholesterol and cerebrosides on BACE activity. *All lipids were of brain origin except for cholesterol (sheep wool). BACE was reconstituted in LUVs of given compositions by detergent mediated reconstitution followed by flotation in a density gradient to separate resulting proteoliposomes from unreconstituted protein. BACE content was determined in each sample, and specific activity of BACE in each particular mixture measured and is expressed in % relative to the specific activity of BACE in total brain lipid protoeliposomes. A, increasing cholesterol:sphingolipid ratio correlates with incresing specific activity of BACE. All three mixtures contained 50% w/w PC, 20%w/w PE and 5% w/w gangliosides. Increasing amounts of cholesterol (indicated in % w/w) were introduced at the expense of the SM content. B, cerebrosides seem to enhance BACE activity. The three mixtures contained 45-50% w/w PC, 20% w/w PE, and 15% w/w cholesterol; in addition, the mixture lacking cerebrosides (light gray bar) contained 5% w/w gangliosides and 10% w/w SM, while the mixtures represented by the dark grey bars contained 10% w/w cerebrosides and the right-most mixture contained additional 5% w/w SM.*

4.4.4. Specific activity of BACE reconstituted in vesicles composed of glycerophospholipids, (glyco)sphingolipids, cholesterol, including negatively charged glycerophospholipids

Mixtures listed in Table 1 will be throughout this paragraph referred to by their numbers enclosed in braces {}.

In pure glycerophospholipid vesicles, anionic phospholipids (PS and PA) promoted BACE activity notably. In vesicles composed of glycerophospholipids, sphingolipids, and cholesterol, the outcome was

dependent on the sphingolipid content of the mixture: In otherwise identical mixtures (either with {3,6,8}, or without {″0″,2,10}, cerebrosides), there was a clear trend of increasing BACE activity with increasing amounts of PS (**Figure 15**). However, in mixtures with different sphingolipid content, the response to the addition of PS varied (compare {2,4,5,6}). Thus the ratio of sphingolipid to phospholipid (or perhaps cholesterol) content may play a role as well. We further asked if cerebrosides contribute to supporting activity in mixtures of PC:PE:±SM:chol:PS. Comparing {0,2,4,10} to {3,5,6,8} revealed higher activities in the mixtures that included cerebrosides.

With PA, the situation is somewhat different. Introducing 5% PA to mixture of PC:PE:SM:cbro:chol promoted activity more notably than 8% PS (compare {7,3,6}). However, when in this case cerebrosides are omitted, the resulting mixture {9} displays high activity.

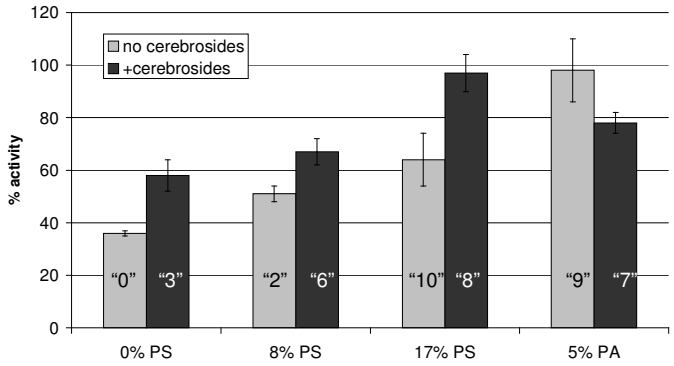


Figure 15 Specific activity of BACE in vesicles composed of PC, PE, cholesterol, SM, ±cerebrosides, ±PS/ ±PA. Anionic phospholipids and cerebrosides affect BACE activity: Activity increases with increasing PS content in proteoliposomes of otherwise identical compositions, and cerebrosides included at the expense of PC in proteoliposomes of otherwise identical compositions further stimulate the activity.

BACE was reconstituted in LUVs by detergent mediated reconstitution followed by flotation in a density gradient to separate resulting proteoliposomes from unreconstituted protein. BACE content was determined in each sample, and specific activity of BACE in each particular mixture was measured and is expressed in % relative to the specific activity of BACE in total brain lipid protoeliposomes (=100%).

In all mixtures, PE is present at a concentration of 18% mol/mol (20% w/w), cholesterol at 26% mol/mol (15% w/w), and SM at 5% mol/mol except for sample "0" (9% SM) and "2" (14 mol % SM). Where applicable, cerebrosides are present at a concentration of 12 mol%. Amounts of PS/PA are indicated. The numbers on the bars indicate the mixture number in ***Table 1.***

4.4.5. Activity in cholesterol-depleted proteoliposomes

Methyl-β-CD is often used for cholesterol depletion from cell membranes. Using this same approach on proteoliposomes allows to distinguish between the requirement for cholesterol to maintain the activity of reconstituted BACE as opposed to the requirement for cholesterol presence during membrane insertion of BACE. When cholesterol was depleted either from the reconstituted proteoliposomes, or from liposomes before BACE insertion by means of methyl-β-cyclodextrin treatment (~5mM), activity of BACE was dramatically reduced (~4-fold) in comparison with untreated control, while there was no effect on non-reconstituted solubilized BACE (Figure 16). This experiment was performed with brain lipids proteoliposomes and soybean lipids proteoliposomes. The total soybean lipids do not contain cholesterol, but contain significant amount of plant sterols that play a role analogous to cholesterol in maintaining membrane properties.

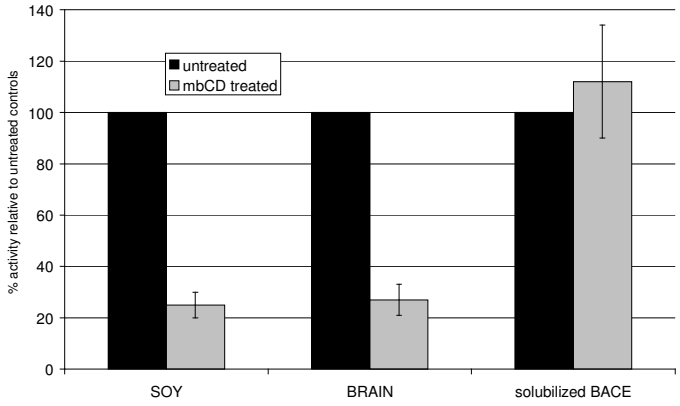


Figure 16 Specific activity of BACE in proteoliposomes after treatment with mβCD. *Proteoliposomes with reconstituted BACE and control (BACE in Triton X-100) were treated with 5mM mβCD for 15min. Activity was then measured after dilution with the assay buffer. Activity in non-treated controls was set to 100%.*

4.5. Reconstitution of APP and BACE into giant unilamellar vesicles

Fluorescently labeled proteins were used for reconstitution into giant unilamellar vesicles (GUVs). BACE and APP were labeled using the amino-reactive dyes Cy5 (excitation maximum 649nm, emission maximum 670nm)

and Alexa 488 (excitation maximum 495nm, emission maximum 519nm) with 200 – 300% efficiency (ie 2-3 fluorophor molecules per protein molecule). The labeling had no influence on activity (data not shown).

Upon reconstitution in LUVs, these large proteoliposomes were fused to form giant, mostly unilamellar proteoliposomes of 5 – 250 μm diameter. The proteo-GUVs were stable for several hours.

4.5.1. Partitioning properties of BACE in giant unilamellar vesicles

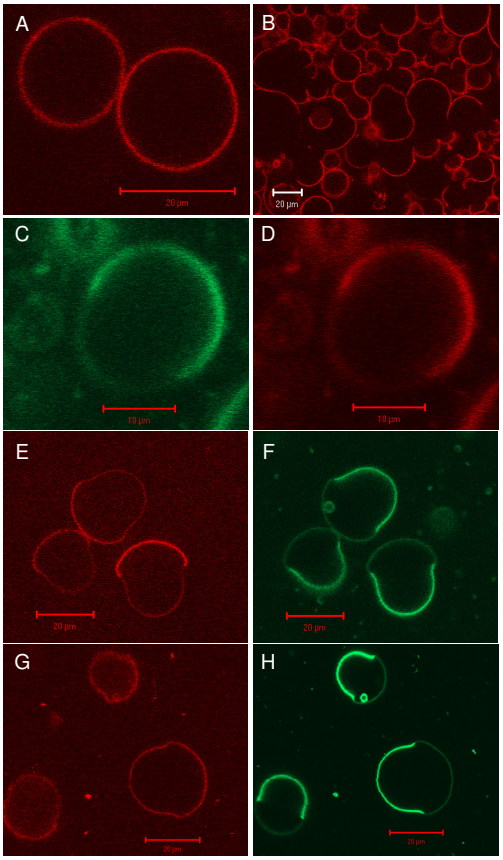


Figure 17 Confocal images of the proteo-GUVs. *Red channel – CY5-BACE, green channel in C – ld marker DiO, green channel in F and H – Alexa488-CTB. A – CY5-BACE in DOPC GUVs; B – overview of CY5-BACE in DOPC:SM:chol (2:2:1 mol/mol); C and D: CY5-BACE in DOPC:SM:chol:DiO (2:2:1 mol/mol + 0.1 mol% DiO) – C: DiO, D: CY5-BACE; E and F: CY5-BACE in DOPC:SM:chol:GM1 (2:2:1 mol/mol+ 0.1 mol% GM1) after cross-linking with CTB; E: CY5-BACE, F: Alexa488-CTB; G and H: CY5-BACE in DOPC:SM:chol:GM1 (2:2:1 mol/mol+ 1 mol% GM1) after cross-linking with CTB – G: CY5-BACE, H: Alexa488-CTB. The scalebar in panels A, B, E, F, G, H is 20μm, in panels C, D 10μm.*

BACE has been previously found to be partially DRM-associated [77], however DRM association does not directly imply raft association. We therefore chose to approach the issue of putative raft association of BACE by probing the partitioning of BACE between the *lo* and *ld* phases in GUVs.

As expected, distribution of BACE in pure DOPC vesicles (*ld* phase only) is homogenous (**Figure 17A**). However, we found that in a system with coexisting *ld-lo* phases BACE does indeed partition into the *lo* phase. In GUVs composed of DOPC:brainSM:cholesterol 2:2:1 mol/mol (this mixture gives rise to large, microscopic domains of several *μm* in diameter), about 15-20% of BACE partitions into the *lo* phase - as judged from the fluorescence correlation spectroscopy measurements and confocal imaging (**Figure 17B**). When 1 mol % of GM1 (or more) is included in the membrane, then upon cross-linking with cholera toxin subunit B, redistribution of BACE occurred, BACE being almost equally distributed between *ld* and *lo* phases (**Figure 17E,F**). This may reflect a specific interaction of BACE with GM1. Presence of GM1 or brain gangliosides at ~2% mol/mol did not lead to any observable change in the specific activity of BACE in large proteoliposomes, however since we are aware of the putative role of GM1 in amyloidogenesis [for example 78,79], the

possibility of engagement of GM1 or other gangliosides directly in regulating *β*-cleavage itself will be further addressed in future experiments focusing on the interaction of BACE with full-length APP rather than with the peptide substrate.

Diffusion coefficients of BACE measured by FCS were 3.5 x 10^{-8} ± 0.5 cm^2s^{-1} in the *ld* phase of 2:2:1 DOPC:brainSM:chol, and 0.7 x 10^{-8} ± 0.1 cm^2s^{-1} in the *lo* phase of the same system.

4.5.2. Partitioning of APP in GUVs

As expected, in a system with co-existing *ld* and *lo* phases (the same system was used as in the experiments involving BACE), the vast majority of APP partitions into the *ld* phase. While about 20% of BACE partitions into the *lo* phase, only less than 5% of APP was estimated to be *lo*-phase associated (**Figure 18**).

APP behaved differently from BACE also in that cross-linking of GM1 (when included in the same system at either 0.1 or 1 mol %) did not influence the partitioning.

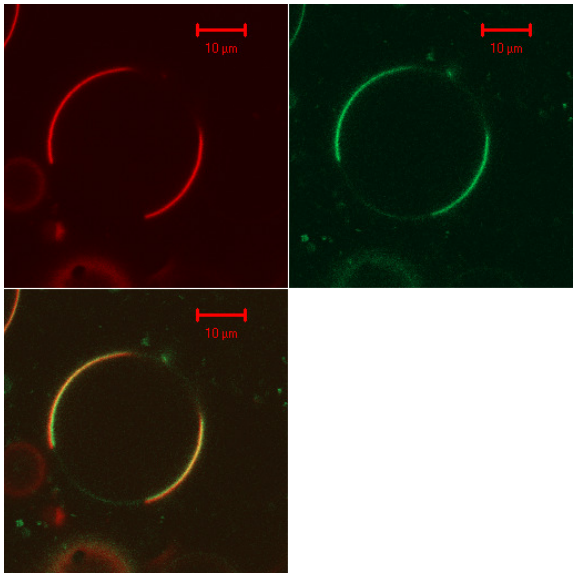


Figure 18 Partitioning of Cy5-APP in GUVs. *More than 95% of APP partitions into the* ld *phase of the system DOPC:SM:chol:DiO (2:2:1 mol/mol + 0.1 mol% DiO). From left to right, top to bottom: red channel, Cy5-APP; green channel, DiO; merge.*

4.6. Co-reconstitution of APP and BACE in large proteoliposomes

Finally, APP was co-reconstituted with BACE in large proteoliposomes (**Figure 19**). The main obstacle was preventing cleavage of APP by BACE during the procedure.

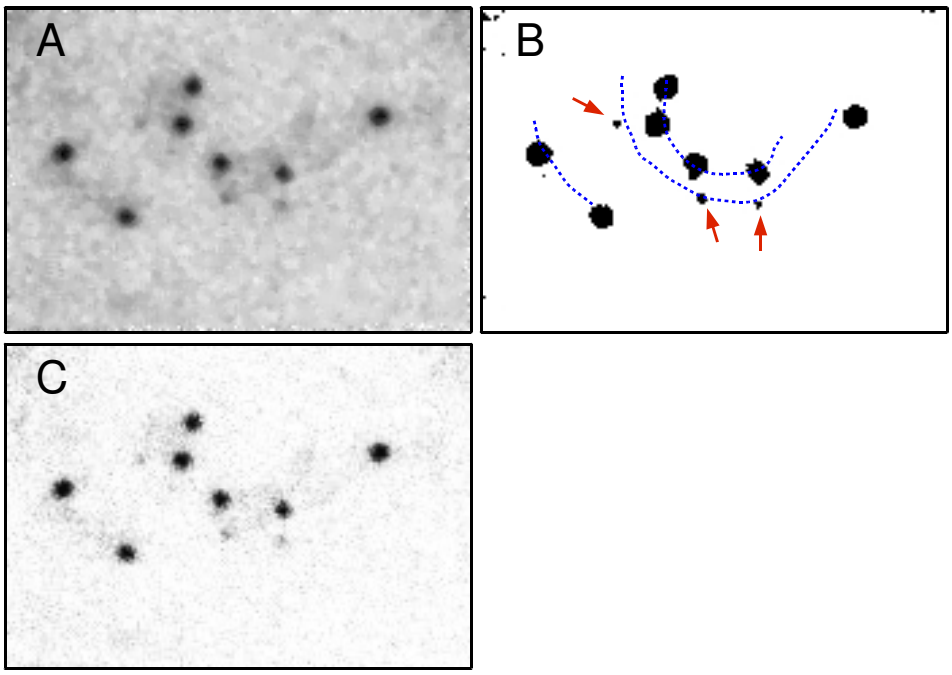


Figure 19 Immunoelectronmicroscopy of the proteoliposomes comprising both APP and BACE.

Detection: APP - 6E10 antibody + 12nm gold conjugated secondary antibody, BACE - 7523 antibody + 6nm gold conjugated secondary. Panel A shows the raw image; the membrane outlines are weakly visible; the large dots correspond to APP, the smaller dots (difficult to see, see panel B) to BACE. Panel B shows a processed image (threshold applied) to make the gold particles stand out over the grainy background. The red arrows indicate the position of the 6nm gold particles, the blue dashed lines copy the contours of the membranes. Panel C shows a digitally processed, enhanced image.

Even though BACE shows very little activity towards the soluble fluorescent peptide at pH higher than 5.5 in solubilized state, the situation is dramatically different in proteoliposomes with full-length APP. Liposomal membranes provide only 2D space for APP and BACE, and thus the probability of BACE "finding and grabbing" APP is very high. In addition, the full-length APP may be a "better substrate" than the artificial peptide. Therefore the procedure used for reconstituting APP or BACE individually had to be modified somewhat for co-reconstituting APP with BACE together. First, the experiments were scaled-down so that smaller volumes were being processed – this shortens the time required for certain steps (gel filtration, centrifugation steps), thus also

limiting the cleavage. Second, the buffers were changed so that directly after the gel filtration step, the proteoliposomes were diluted into a buffer of pH 8 – 8.2. In addition, all centrifugations were performed at 2°C. In spite of all these precautions, it was not possible to prevent the cleavage completely.

4.7. Reconstitution of the β-secretase activity of BACE in proteoliposomes: cleavage of APP by BACE in proteoliposomes

APP and BACE were co-reconstituted in proteoliposomes. The objective was to reconstitute the initial step of the amyloidogenic processing of APP (the β-cleavage) in an environment mimicking the native environment – the lipid bilayer. The β-cleavage of APP was detected in proteoliposomes with APP and BACE reconstituted together at various ratios, but not when BACE was omitted (there is no detectable spontaneous β-ectodomain generation) Figure 20.

Next, it was important to investigate whether solubilized full-length BACE or the soluble BACE ectodomain are capable of β-processing of the membrane-embedded APP, which does not seem to be the case (data not shown).

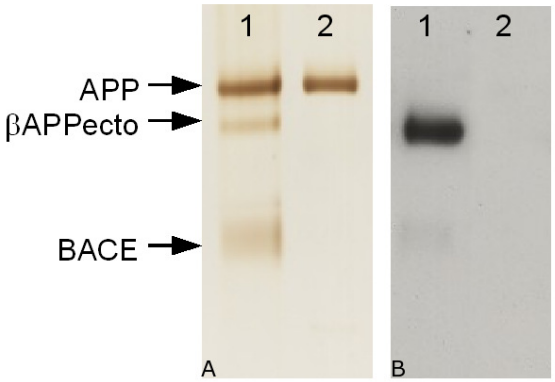


Figure 20 Full-length APP co-reconstituted with BACE in total brain lipid proteoliposomes is cleaved at the β-cleavage site. *Purified full-length APP was co-reconstituted with BACE in total brain lipid proteoliposomes and incubated at pH 7 at room temperature. Proteoliposomes were then loaded on SDS-PAGE gels (lane 1) along with control liposomes containing APP only (lane 2). Panel A shows a silver stained gel, panel B shows a western blot of a similar gel probed with anti-BACE antibody and an antibody specifically recognizing the β-cleaved ectodomain of APP.*

While for BACE activity assays with the amyloidomimetic peptide substrate the optimal pH was ~4.8, it appears that the full-length APP is unstable in an acidic environment and it degrades rather fast at pH 5, as mentioned in paragraph 4.2.1. Thus in the artificial purified system, at low pH conditions, the β-secretase substrate is being non-specifically depleted. For this reason, all activity assays with the proteoliposomes comprising the full-length APP were performed at pH 6.6 – 6.8.

4.7.1. β-processing of the full-length APP in proteoliposomes of different lipid compositions

To follow the β-cleavage, an assay for detection of the β-cleaved ectodomain of APP was developed. The assay takes advantage of the MSD technology, (described in 3.2.11). Several capture/detection antibody combinations were tested before arriving at the current setup. Since it is impossible to completely quench BACE activity without denaturing the samples, the key requirement for both capture and detection antibodies was that they need to recognize denatured APP. The present procedure for preparing the sample for analysis thus includes solubilization of the proteoliposomes with high pH buffer (final pH 7.8) containing SDS/NP 40 detergent combination (SDS also deactivates BACE). In addition, the solubilized samples are shortly heated to 95°C. This treatment ensures good pick-up of both the full-length APP and the cleaved ectodomain by the capture antibody; only the β-cleaved antibody is however detected owing to the specificity of the detection antibody. Initially, a setup in which only the β-cleaved ectodomain was captured had been tested, but was later dismissed because of the problems caused by direct binding of the full-length APP to the surface of the wells. With the current assay, approximately 1ng/mL of the denatured β-cleaved ectodomain can be detected in 25μl total volume (which corresponds to 25pg total ectodomain).

To quantitatively analyze the kinetics of the β-processing of APP in proteoliposomes is, however, a difficult task. While it is relatively straightforward and painless to analyze enzyme kinetics with soluble enzyme and substrate, when one is able to independently change the concentrations of both enzyme and substrate, studying kinetics of cleavage in proteoliposomes is substantially different. First, with proteoliposomal sample, one does not have a full control over the amount of enzyme and substrate in the reaction mix: choosing a working concentration of the substrate automatically predetermines

the concentration of the enzyme and *vice versa* (varying the APP:BACE ratios during reconstitution of course does affect the ratio in the resulting proteoliposomes, however, the result is not predictable with 100% accuracy). Furthermore, due to the fact that APP and BACE are being co-reconstituted, the zero timepoint of the reaction is not a bona fide **zero** point, because a certain fraction of the substrate had already been converted during the reconstitution procedure. Therefore, in order to compare enzyme activities in different samples, a number of requirements concerning the proteoliposomes samples have to be fulfilled. First, a need for favorable APP : BACE ratio. Ideally, the enzyme should be saturated with the substrate, i.e. the substrate should be present in great excess over the enzyme. Since this does not seem to be possible to achieve with the current method, samples with similar ratios and total amounts of APP and BACE were used. Second, the conversion at timepoint zero (set as a moment of addition of the reaction buffer) should be similar, and negligible relative to the total amount of APP present. Third, the inter-vesicular distribution of APP and BACE, as well as the total protein-to-lipid ratio (which is a measure of the total protein surface density, and thus is analogous to 3D concentration) has to be comparable. Keeping all these restrictions in mind, it is a major problem to find samples fit for comparison.

Great care was taken to use suitable samples of total brain lipid proteoliposomes and POPC proteoliposomes, and enzyme progress curves were recorded. Since it turns out that the quantification of the APP and BACE content by dot-blotting may be burdened with a considerable error, the current results should be regarded as semi-quantitative only.

The results indicate that BACE co-reconstituted with APP in POPC proteoliposomes performs reproducibly poorer than in total brain lipid proteoliposomes. A representative pair of reaction progress curves is shown in Figure 21. In samples with approximately two-fold excess of APP over BACE, this and other experiments indicated the halftime of the β-cleavage in total brain lipids to be in the range of 0.5 - 4 minutes (2 minutes for the case shown here), while in POPC vesicles much longer times were needed, generally exceeding 5 minutes (17 minutes for the case shown here).

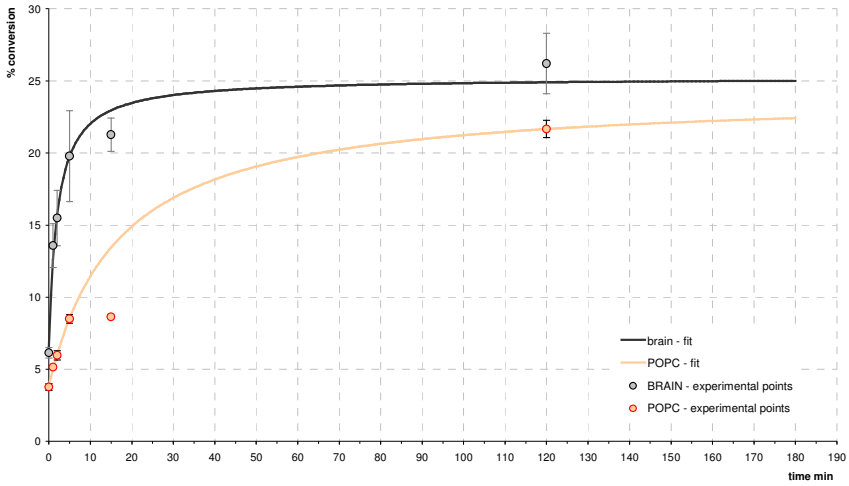


Figure 21 Reaction progress curve for APP cleavage by BACE in brain lipids and POPC proteoliposomes. *The two samples had essentially identical content of APP and BACE (as judged by dot blot), with APP-to-BACE ratio of about 2. Reactions were done in citrate/phosphate buffer pH 6.6. Samples were removed at relevant timepoints, the reaction was stopped by adding SDS/NP40-containing buffer pH 8.8, and samples were heated shortly to 95°C. The β-cleaved ectodomain was then determined using the MSD assay. The fitted curves are displayed only to indicate the trend of the reaction progress; the actual fit does not analytically describe the real process (general saturation model with offset was used). See the footnote♥ and discussion for more information on fitting reaction progress curves.*

♥ Fitting reaction progress curves is not trivial. In the case of a standard enzyme reaction following the classical irreversible Michaelis-Menten scheme, integrating the fundamental equation $dP/dt = (Vlim*S)/(Km+S)$ [where P denotes the momentary product concentration, S the momentary substrate concentration, and Vlim and Km are the maximal velocity and Michaelis constant, respectively] leads to an equation with implicitly expressed P: $P-K*\ln(1-P/S_0)-Vlim*t=0$ [S_0 denotes the initial substrate concentration; then obviously $P= S_0-S$]. It has been shown that no algebraic arrangement of this equation can convert the expression into an expression suitable for standard regression analysis. Specialized software for fitting curves to implicit functions is required. Another option is parameter optimisation using the integral version of the equation, ie $P=INT(Vlim*S/(Km+S))dt$. Like in the case of implicit functions, this approach requires specialized software tools. Even though not as convenient as standard curve fitting, either of the two approaches is feasible. However, in comparison to standard regression, these methods can only yield reasonable results provided that the dataset consists of sufficient number of experimental points with minimal error and precisely defined initial conditions (which is not the case of the presented data). Another problem in this particular case of cleavage of APP co-reconstituted with BACE in proteoliposomes is that this process may not obey the simple Michaelis-Menten model. See Discussion for more information.

5. Discussion

Membrane proteins are designed to reside and function in lipid bilayers and hence the properties of the bilayer affect the biological activity of the associated proteins.

Being an integral membrane protein, the aspartic protease BACE is likely to respond to its lipid environment on a molecular level (conformation, oligomerization, intramolecular charge distribution etc.) in a way that could eventually affect its activity. BACE activity is in cells directed towards another integral membrane protein, APP, which is a precursor of the neurotoxic amyloid peptide (Aβ). Previous work had indicated that production of the Aβ peptide may be linked to lipid metabolism and composition of cell membranes. In this thesis, the issue of involvement of membrane lipids in modulating β-secretase activity was addressed.

In order to experimentally address this issue, BACE and APP were purified and reconstituted in proteoliposomes of defined lipid compositions, and the influence of the lipid composition on BACE activity was analyzed.

First, we investigated the dependence of the proteolytic activity of BACE irrespectively of APP as **the** substrate, but using a soluble peptide substrate, on the composition of the lipid bilayer in a reconstituted system consisting of purified full-length BACE and defined lipids.

Activity of the soluble ectodomain of BACE is lower than that of the full-length protein

Even though the soluble BACE ectodomain alone displays proteolytic activity, a higher specific activity of the full-length BACE has been reported [80]. It was proposed that the higher specific activity of the full-length protein may be due to dimerization, which would occur exclusively when the ectodomain is attached to the membrane. Using BN-PAGE, we have detected dimers of the purified recombinant ectodomain of BACE as well as oligomers of the full-

Oligomriza-tion of BACE

length BACE (data not shown). Dimerization thus does not seem to be strictly dependent on membrane attachment, which may nevertheless promote self-association, and if that is the case then chances are that certain lipids support dimerization or stabilize dimers better than other. This issue was however not pursued any further since straightforward but adequate methods for analysis of oligomerization of membrane proteins are not readily available. While it is relatively painless to investigate oligomerization of a soluble protein (by gel filtration, velocity gradient centrifugation and other basic methods), with membrane proteins the situation is very different. In principle, all methods that can be used on soluble proteins can also be used on **solubilized** membrane proteins, however it is not clear whether the results concerning oligomerization of detergent-solubilized membrane protein are relevant to its native, mambrane-embeded state. Methods allowing evaluation of oligomerization of proteins directly in membranes are few, require expertise, and are generally experimentally demanding. These methods include FRET, cross-correlation analysis of the labeled proteins, chemical cross-linking and other [reviewed in 81].

Analyzing oligomeric state of membrane proteins

Importantly, upon reconstitution of the full-length BACE in proteoliposomes, we observed further increase in specific activity (full-length solubilized BACE displayed a higher activity than the ectodomain alone, but full-length membrane-embedded BACE was more active than the full-length solubilized BACE). Thus, it appears that neither oligomerization nor the absence of potentially inactivating detergent can fully explain this increase in specific activity, because the increase was also found to depend on the **lipid composition** of the proteoliposomes.

Activity of reconstituted BACE depends on the lipid composition

We had hypothesized that lipids differ in their capacities to support enzymatic activity of BACE. Our data 1/ back up the concept of a multiple-lipid system being generally more capable of supporting protein activity, 2/ suggest lipid candidates involved in affecting BACE activity. We focused on investigating effects of lipids defined by their headgroups, and did not examine impact of acyl chain composition.

Anionic phospho-lipids

All cell membranes comprise anionic glycerophospholipids - PA, PIs, and PS, the latter two being confined almost exclusively to the cytoplasmic leaflet of the membrane. Negatively charged lipids have been identified as regulators of function of numerous integral membrane proteins (glucose transporter, Ca^{2+}-ATPase, (Na^{+}, K^{+})-ATPase [82 and references therein], nicotinic acetylcholinesterase receptor [82 and references therein, 83]).

We have demonstrated a strong effect of PS and PA on BACE activity, which is further enhanced by PE. Considering the fact that anionic glycerophospholipids localize mainly to the cytoplasmic leaflet of the membrane, and that the catalytic domain of BACE is localized on the other side of the membrane, it is clear that especially in context of the experiments performed with artificial soluble peptide which may interact with the membrane surface, it is necessary to interpret these results carefully. We can envisage four possible, mutually not exclusive, ways, in which PS or PA could encourage proteolysis by BACE: electrostatic interactions at the membrane interface, a direct interaction of BACE with a negatively charged lipid in the outer leaflet, a direct interaction of the cytoplasmic tail in the inner leaflet of the proteoliposome, and anionic lipids influencing the bulk membrane properties other than electrostatic.

Anionic phospholipids: Electrostatic effects

Local electrostatic effects may result in changes in local ion concentrations (charged peptide substrate, H^+ - local decrease in pH). Electrostatic interactions involving anionic glycerophospholipids (which account for ˜30% of cellular phospholipids) belong to the fundamental principles of biological interactions at membrane interfaces. Common characteristic of these non-specific events is the requirement for a minimum interfacial concentration (˜10mol%) of the anionic phospholipid before an effect is observed [84]. Since we see a striking effect already at a relatively low concentration of PA (5%), it is not clear whether this is the case in our system, where externally exposed anionic phospholipids could be responsible for the recruitment of the peptide substrate to the membrane. In order to avoid complications of this type, it would be necessary to employ proteoliposomes with asymmetrically distributed lipids, restricting the anionic lipids to the inner side to mimic the topology of cell membranes. However, such systems have not yet been used for membrane protein studies, even though several promising attempts to produce asymmetrical liposomes have emerged [85, 86].

Under ionic strength conditions much lower than in our system (5 - 10mM), the local pH near the surface of the membrane containing anionic phospholipids is 1.5 - 2 pH units lower than in bulk solution [87]. Optimal pH for BACE activity is ˜4.5, the activity steeply dropping above pH 5.5. If the local pH effects caused by the presence of PS/PA were the major mechanism for stimulating BACE activity in proteoliposomes, one would expect the decline in activity of reconstituted BACE to be less steep with increasing pH, which was not the case (not shown).

Anionic phospholipids: Direct interaction

Another way in which anionic lipids may be involved in stimulating BACE activity is a direct interaction of BACE with a negatively charged species in the outer leaflet, resulting in changes in BACE conformation, oligomeric state, or charge distribution. The only negatively charged species continuously and extensively present in the outer leaflet of the plasma membrane are charged glycosphingolipids (gangliosides and sulfatides). Role of gangliosides and sulfatides was not thoroughly investigated in this study. Small amounts of gangliosides (5% w/w, 2% mol/mol) used in mixtures with PC, PE, SM, and cholesterol, did not cause any observable change in activity. In addition, an interaction of the cytoplasmic tail with PS or PA (or PI which was not experimentally tested in this study) in the inner leaflet could as well affect the conformation of the protein. Anionic phospholipids have also been shown to strongly influence other bulk membrane properties, including lipid packing density and perturbing the bilayer structure [84]. This phenomenon can account for facilitated penetration of the transmembrane domain, resulting in increased stability of BACE insertion, eventually affecting its activity.

Glyco-sphingo-lipids stimulate BACE activity

As opposed to anionic phospholipids, glycosphingolipids mainly localize to the outer leaflet of the plasma membrane, and brain tissue in particular has been found to contain high amounts of neutral glycosphingolipids - cerebrosides. Brain cerebrosides contain mostly long saturated fatty acid chains (C22:0, C24:0, and also C24:1 (information from Avanti catalog)). The stimulatory effect of cerebrosides in proteoliposomes on BACE activity may be owing to these long acyl chains or may be due to the presence of carbohydrate headgroups that besides thickening the layer of perturbed water near the membrane interface may interact with the BACE ectodomain. Interestingly, cerebrosides also activate soluble BACE lacking the transmembrane and cytosolic part, which further supports the possibility of saccharide headgroup interacting with the BACE ectodomain.

Implication of rafts and the liquid-ordered phase in regulation of BACE activity

BACE partitions in *lo* phase in GUVs

Both APP and BACE have been suggested to associate, to a certain extent, with lipid rafts, and thus rafts had been implicated in β-cleavage regulation. Since our hypothesis assumes β-cleavage of APP to happen primarily in lipid rafts, in order to examine the possibility of BACE requiring *lo* phase domain to assume a high-activity state, we used liposomes consisting of PC:SM:chol (2:2:1 or 1:1:1 mol/mol, there was no significant difference when either POPC or DOPC was used), which has been shown to phase separate at relevant temperatures [88]. Even though in GUVs BACE does not show a preference for the *lo* phase, the measured 15-20% seems to be a significant fraction when

compared to other proteins that have been probed using the same technique. 25-30% of the GPI-anchored placental alkaline phosphatase (PLAP), which has become a ″prototype of a raft protein″, and is recognized as a raft marker, partitions into *lo* phase in the same system [89], and also only 10 - 20% of a LAT-derived peptide was reported to partition in *lo* phase. On the other hand, syntaxins or bacteriorhodopsin partition almost exclusively (95-99%) into *ld* (N. Kahya, personal communication). Interestingly, partitioning of BACE into *lo* phase seems to be enhanced by cross-linking of the membrane components (we have used the pentavalent CTB to cross-link GM1), offering potential means of regulating BACE-raft association in cells (of course not via cross-linking with CTB, but perhaps thanks to binding of other, endogenous lectin to membrane components, or directly to BACE). These experiments therefore further back up the hypothesized ability of BACE to (dynamically) associate with rafts (*lo* phase), possibly in a flexible, adjustable manner, which is a feature compatible with the role of BACE as a regulated protease. Interestingly, only negligible, barely detectable amounts of APP were found to partition in *lo* phase in the same system, and cross-linking of GM1 with CTB did not affect its *ld*-*lo* distribution. Does this mean that the *β*-cleavage does not happen in rafts after all? This question is difficult to answer considering current data. It is important to emphasize that the extent to which BACE associates with *lo* phase in GUVs is by no means usual for a transmembrane protein. Therefore it seems reasonable to consider this fact significant and potentially physiologically relevant. It is possible that our system is missing a component necessary for APP to find its way to the *lo* phase; our composition PC/SM/cholesterol (with only minor GM1 amounts) only very roughly mimics physiological conditions (complexity of lipid composition, leaflet asymmetry). APP may require a certain lipid (or protein) to change its partitioning preferences. Clearly, more experiments are needed to elucidate this issue; such experiments are still on the schedule, including cross-linking of APP with antibodies or a lectin, as well as improving lipid composition of the GUVs to better imitate physiological conditions while still providing coexisting *ld/lo* phases.

APP does not partition into *lo* phase in GUVs

Unfortunately, GUVs with both APP and BACE were of poor quality not suitable for detailed analysis by FCS; the preliminary results (from imaging) do not indicate any differences in partitioning from the systems with individually reconstituted proteins.

Cholesterol

Even though neither cholesterol alone, nor the *lo* phase in the system *per se* can account for full activation, cholesterol levels clearly influence BACE activity. We show correlation between cholesterol levels and BACE activity in mixtures including PC, sphingolipids and PE. While a direct interaction of BACE with cholesterol (BACE was found to label strongly with photocholesterol in the native environment of the cellular membranes) cannot be excluded, it seems more probable that cholesterol content primarily affects the bulk properties of the liposomal membrane. In accord with this observation is the steep drop in activity of BACE in both asolectin (contain plant sterols) and total brain lipid proteoliposomes upon treatment with methyl-β-cyclodextrin.

Conclusion I
Anionic GPL, GSL, and cholesterol stimulate BACE activity

It should be noted that our experimental approach provides information on the **mean specific activity** of BACE in proteoliposomes of a particular **average lipid composition**. We cannot distinguish between a situation where a part of the pool of the reconstituted BACE would be completely inactive, and the rest present in a highly activated state (which may be the case in bilayers displaying lateral domain separation), from a situation where all BACE molecules would display the same activity. Despite this limitation, we have identified 3 classes of lipids affecting BACE activity in proteoliposomes: 1. anionic glycerophospholipids (whose stimulating effect is potentiated by PE), 2. (neutral) glycosphingolipids (cerebrosides), 3. sterols (cholesterol).

All lipids are capable of affecting multiple properties of the membrane, and obviously, properties of individual lipid species are not combined by simple additive principles (note for example the effect of PE in combination with PS, but not alone, effect of cholesterol, or the effect of cerebrosides). Without understanding principles of lateral lipid organization, and without means to control vertical lipid organization in artificial systems, we cannot completely mimic the conditions in the native environment.

From simple system to reconstitution of the real β-cleavage

Nevertheless, using the soluble peptide substrate, we demonstrated that classical raft lipids - cerebrosides and particularly, cholesterol - promote activity of BACE in proteoliposomes. So far, all results that have been discussed had been obtained with the simplistic experimental set-up involving cleavage of soluble artificial substrate by reconstituted BACE. Such simplification is useful to identify lipids influencing solely BACE as an enzyme, but cannot reveal how the β-processing may be affected due to an interaction of a lipid with APP or owing to the partitioning of APP and BACE in raft-like domains. To recapitulate the mechanisms by which lipids could be involved in

regulation of the β-cleavage (**Figure 22**): 1. lipids as directly interacting partners for either APP or BACE, 2. lipids shaping the overall bulk properties of the membrane and thus indirectly influencing the state (for example conformation) of either APP and BACE, 3. lipids determining the domain organization of the membrane and hence providing means to bring APP and BACE together or to keep them apart, 4 - extending point 3: facilitating trafficking of BACE and APP within the cell.

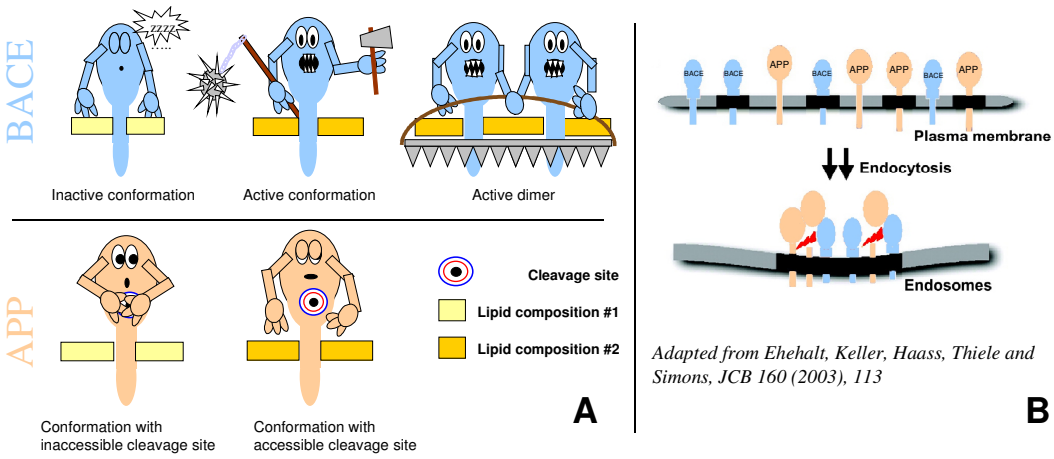


Figure 22 Possible modes of action of lipids on APP and BACE. *Panel A - particular lipid species or particular lipid composition may support certain conformational state of APP/BACE, Panel B - lipid rafts had been suggested to serve as interaction platforms for APP and BACE, mediating their coming together and facilitating endocytosis*

Rafts, endocytosis, and the β-cleavage in cells

The fourth point had been addressed previously in this lab [51], and has been the main influence for the formulation of the hypothesis implicating rafts in β-processing. In this study it has been shown that under the conditions of inhibited endocytosis, the β-cleavage is reduced, however cross-linking of APP and BACE can overcome this reduction, suggesting that clustering rafts containing APP and BACE on the cell surface can mimic their coming together somewhere down the endocytic pathway. Even though fairly clear-cut results at a first sight, when analyzed further, several ambiguities surface. The finding that most of the β-cleavage happens upon endocytosis was in accord with several other studies arguing for the requirement of endocytosis for the β-processing, however studies claiming the opposite emerged as well [14]. There are several reasons that can explain the discrepancies, some of them inherent to studies on cells in culture in general. (overexpression of the involved proteins and lack of means to decide between direct and indirect effects), and some specific for the β-processing study; these include the different approaches taken to prevent endocytosis, different methodology to detect the cleavage and

neglecting the degradation of the β-cleaved products. In addition, even the elegant experiments presented in [51] allow for ambiguous interpretation, since one cannot exclude that cross-linking APP and BACE in a theoretical environment totally devoid of rafts would nevertheless lead to increased cleavage especially under the conditions of overexpressed APP. Therefore in order to implicate rafts in cleavage, it may be useful to uncouple the β-processing step from cellular physiology.

Proteoliposomes of different lipid compositions, either with or without phase-separated co-existing *lo* domains, seem to be an ideal tool to answer questions addressing the role of lipid rafts in β-processing. While the role of raft lipids (and the role of phase-separated *lo* domains mimicking the raft environment in liposomes) cannot be revealed by probing BACE activity with the soluble substrate, it is likely to become obvious when BACE activity is probed towards its natural substrate, the co-reconstituted full-length APP.

BACE cleaves APP in proteolipo-somes at the correct site.

We have established that BACE cleaves the co-reconstituted APP in total brain lipid liposomes at the correct site, proving our reconstituted system to be relevant and well suited for testing the hypothesis of raft-mediated β-cleavage of the full-length APP.

Cleavage in proteolipo-somes of different compositions

To begin with, we have asked a simple question: is there a detectable difference in rate of APP cleavage by BACE in total brain lipid liposomes and POPC liposomes?

Setting up the **in vitro** cleavage assay

Conceptually simple experiment, yet technically not trivial to set up. Such assay poses several problems.

First, unlike ″every other″, usual enzymatic *in vitro* assay, in this assay substrate and enzyme are not added separately, but they come together embedded in liposomes; thus, one cannot independently control the enzyme and substrate concentration*. Multiple samples were therefore prepared, content of APP and BACE was evaluated by dot blotting, and comparable samples were used for comparative activity assays.

Second, since the protease and the substrate are present together from the moment of starting the reconstitution procedure, cleavage can happen at any stage during the procedure or storage. To a large extent, we have overcome the problems of premature cleavage by optimizing the reconstitution procedure

* one possible way to circumvent this problem would be to use 2 different populations of proteoliposomes, one population with APP, and the other with BACE, and fuse them; however, this will only be possible when efficient methods for liposome fusion are available.

(the parameters that are of highest importance are speed, pH, and temperature); nevertheless, this unwanted proteolysis could not be totally quenched, which leads to uncertainty about timepoint zero conditions in our assay, which is a crucial parameter for kinetic analysis. Third and perhaps most critical, achieving favorable APP-to-BACE ratio in proteoliposomes is essential.

Analyzing enzyme reaction data

The rate of an enzyme-catalyzed conversion of a substrate to a product depends on the concentration of the substrate, but only until a certain threshold concentration is reached. This threshold corresponds to the so-called enzyme saturation. Enzyme saturation is reached when essentially all the enzyme is present in the form of enzyme-substrate complex. Under these conditions, the rate of conversion holds constant until a point when the substrate is depleted below the level necessary for enzyme saturation, and the apparent activity of the enzyme thus decreases. Hence, it is possible to compare specific enzyme activities in different samples by comparing the reaction rates only when the enzyme is saturated; in this situation small differences in substrate concentrations do not alter the results. When it is not possible to ensure enzyme saturation, it is necessary to work with samples with comparable enzyme and substrate concentrations. It is however important to point out that in contrast to conventional *in vitro* enzyme assays in solution (3 dimensions), where small deviations from ideal concentrations lead to small effects, in our assays, where the enzyme and substrate are confined to 2D space, at concentrations in the ″nano-scale“ working range (generally the proteoliposome samples contain no more than 20 molecules per vesicle), a small difference may lead to dramatic consequences. A typical example of such behavior is a situation where BACE content corresponds to an average of 1 BACE molecule per vesicle. Since the BACE distribution throughout the vesicle population is not even, but most likely corresponds to the Poisson distribution, this means that there will be vesicles totally lacking BACE as well as vesicles with relative BACE excess. This will in the end effect lead to kinetic parameters that will describe averaged parameters of these different subpopulations of vesicles and this may lead to considerable error in evaluating BACE activity. In addition, as mentioned above, one has to bear in mind that the present study was based on assays with samples harboring minute amounts of the proteins, and it has been shown and studied before that some of the laws

that are assumed to be relevant in the ″conventional″ enzyme kinetics are not obeyed in small-scale, 2D systems♥.

Conclusion II BACE cleaves APP faster in total brain lipid liposomes compared to POPC liposomes

We have been aware of these limitations and pitfalls, and the assays were done with great care to minimize all the maladies. Results that have been obtained to date are encouraging, indicating a slower rate of cleavage of APP in POPC liposomes as compared to total brain liposomes. However, while the experiments proved to be reproducible in that the total brain liposomes were superior to POPC in terms of rate of the β-cleaved-ectodomain production, the saturation levels and the shapes of the enzyme progress curves were less reproducible. At this stage it would therefore be dangerous to pronounce any truly quantitative statements about kinetic parameters in brain lipids and POPC.

Problems and how to solve them: future experiments

Which are the concrete problems and how to solve them? It appears that one serious, yet relatively easy to overcome source of error is the insufficient characterization of the initial proteoliposome preparation - it seems that the current methodology employed for initial protein quantification was inadequate (dot blotting) and that using a more sensitive method (like MSD) would improve the quality of the cleavage assays enormously, and render them more quantitative. Also characterizing the inter-vesicular distribution of APP and BACE (for example by EM) in a quantitative manner may be required as a routine step in the assay.

Nevertheless, the fact that there is a detectable difference between the two, in a way extreme, lipid compositions (single component versus multiple component) is a highly important finding.

Outlook.

Impact of domain organization on the kinetics of β-cleavage

Thanks to the current rapid progress in characterization of ternary lipid mixtures of PC, SM, and cholesterol [90], it seems to be possible to experimentally address the issue of impact of the size of the domains on the kinetics of β-cleavage. It is likely that if rafts/domains mediate the reaction, then the size of the domains, as well as the total amount of each phase, will be important parameters influencing the kinetics, as the kinetics of the reaction itself cannot be uncoupled from the kinetics and rate of encounters between APP and BACE, and these in turn depend on frequency of partitioning in and

♥ The foundation of enzyme kinetcs, the Michaelis-Menten formalism for enzyme reactions, is based on mass-action laws. Mass-action laws are mean-field approximations because they evaluate local reaction rates on the basis of average values of the reactant density over a large spatial domain. They rely on strict assumptions concerning, for instance, the characteristics of the reaction medium, which must be dilute, perfectly-mixed, **three-dimensional**, and homogenous. Many of the required assumptions fail in the case of biological reactions and reactions in 2D [92].

out of such domains and on the residency time within. **Figure 23** presents a possible scheme of controlling the size of the domains in liposomes.

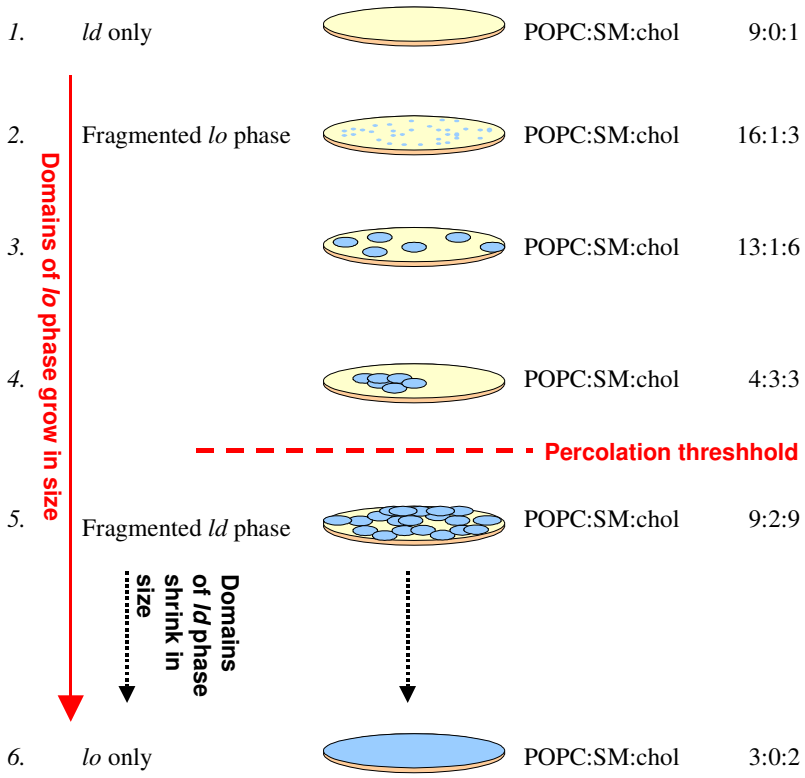


Figure 23 Size of the domains depends on lipid composition of the liposomes. *The figure was conceived based on the data extracted from* [90]. *The lipid compositions are given as molar ratios of the 3 components. Domain size as well as total amount of the phases is likely to have profound effect on kinetics of any raft/*lo*-domain mediated enzymatic reaction.*

Another important area to explore is the issue of cross-linking and the effects it may bring about. The present system - phase separating proteoliposomes comprising a ″raftophilic″ enzyme (BACE) and its substrate (APP), which, on its own, does not seem to display any significant raft-partitioning ability, appears to be a powerful tool for studying phenomena affecting partitioning of the incorporated proteins.

Considering the growing number of studies contributing to deciphering the complicated picture of how proteins and lipids diffuse and interact in biological membranes [69, 91, 92], it is clear that this issue has become one of the mainstreams of cell biology research, and thus we can hope that this increased interest will accelerate our uncovering of how lipids of the cell membranes may be involved in contributing to the development of Alzheimer‘s disease, and how can we make use of this knowledge in medical research.

Some of the results presented in this thesis appear in

Kalvodova L, Kahya N, Schwille P, Ehehalt R, Verkade P, Drechsel D, Simons K. (2005) Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids *in vitro*. *J Biol Chem* **280**, 36815-23

6. References

1. Michael G. Kornacker, Zhihong Lai, Mark Witmer, Jianghong Ma, Joseph Hendrick, Ving G. Lee, Douglas J. Riexinger, Claudio Mapelli, William Metzler, and Robert A. Copeland *Biochemistry* (2005) **44**, 11567-11573
2. Selkoe DJ, Schenk D. (2003) *Annu. Rev. Pharmacol. Toxicol*. **43,** 545–84
3. Selkoe DJ (2001) *Physiol Rev* **81**, 741
4. Schmitz · R. Tikkanen · G. Kirfel · V. Herzog *Histochem Cell Bio*l (2002) **117,** 171–180
5. Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, and Muller-Hill B (1987) *Nature* **325**, 733
6. Okamoto, T., et al. (1995) *J Biol Chemistry* **270**, 4205
7. Ermekova, K. S., Chang, A., Zambrano, N., de Candia, P., Russo, T. and Sudol, M. (1998) *Exp. Med. Biol*. **446**, 161-180.
8. Goldstein LS (2001) *Proc. Natl. Acad. Sci.* USA **98**, 6999-7003
9. Annaert W, De Strooper B (2002) *Annu. Rev. Cell Dev. Biol*. (2002) **18**, 25–51
10. Bouillot C, Prochiantz A, Rougons G, Allinquant B (1996) *J Biol Chem* **271,** 7640–7644
11. Tienari PJ, De Strooper B, Ikonen E, Simons M, Weidemann A, Czech C, Hartmann T, Ida N, Multhaup G, Masters CL, Van Leuven F, Beyreuther K, Dotti CG (1996) *EMBO J.* **15,** 5218-29
12. Christian Haass, Edward H. Koo, Anja Capell, David B. Teplow, and Dennis J. Selkoe (1995) *J Cell Biol* **128**, 537
13. Ruth G. Perez, Salvador Soriano, Jay D. Hayes, Beth Ostaszewski, Weiming Xia, Dennis J. Selkoe, Xiaohua Chen, Gorazd B. Stokin¶, and Edward H. Koo (1999) *J Biol Chem*, **274**, 18851-18856
14. Chyung JH, Selkoe DJ (2003) *J Biol Chem*, **278**, 51035-51043
15. Ehehalt R, Keller P, Haass C, Thiele C, and Simons K (2003) *J Cell Biol* **160**, 113
16. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) *Annu. Rev. Cell Biol*. **9**, 129-161
17. Yamazaki, T., Koo, E. H. and Selkoe, D. J. (1996) *J. Cell Sci.* **109**, 999 –1008

18. Kang, J., Lemaire, H., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K., Multhaup, G., Beyreuther, K. & Muller-Hill, B. (1987) *Nature* **325,** 733-736
19. Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L. and Beyreuther, K. (1989) *Cell* **57,** 115-126
20. Hooper, N. M., Karran, E. H., and Turner, A. J. (1997) *Biochem. J*. **321**, 265-279
21. Blobel, C. P. (2000) *Curr. Opin. Cell Biol*. 12, 606-612
22. Koo, E. H., Squazzo, S. L., Selkoe, D. J. and Koo, C. H. (1996) *J. Cell Sci.* **109**, 991-998
23. Kuentzel, S. L., Ali, S. M., Altman, R. A., Greenberg, B. D. and Raub, T.J. (1993) *Biochem. J.* **295**, 367-378
24. Sambamurti, K., Shioi, J., Anderson, J. P., Pappolla, M. A. and Robakis, N. K. (1992. *J. Neurosci. Res.* **33**, 319-329
25. Van Nostrand, W. E., Schmaier, A. H., Farrow, J. S. and Cunningham, D.D**.** (1990) *Science* **248**, 745-748
26. Allinson, T.M., Parkin, E.T., Turner, A.J. and Hooper, N.M. (2003) *J. Neurosci. Res.* **74**, 342-352
27. Buxbaum JD, Liu KN, Luo Y, Slack JL, Stocking KL, Peschon JJ, Johnson RS, Castner BJ, Cerretti DP, Black RA (1998) *J Biol Chem.* **273,** 27765-7
28. Bennett BD, Denis P, Haniu M, Teplow DB, Kahn S, Louis JC, Citron M, Vassar R. (2000) *J Biol Chem.* **275**, 37712-7
29. Creemers JW, Ines Dominguez D, Plets E, Serneels L, Taylor NA, Multhaup G, Craessaerts K, Annaert W, De Strooper B. (2001) *J Biol Chem.* **276,** 4211-7
30. Shi, X.-P., Chen, E., Yin, K.-C., Na, S., Garsky, V. M., Lai, M.-T., Li, Y.-M., Platchek, M., Register, R. B., Sardana, M. K., Tang,M.-J., Thiebeau, J., Wood, T., Shafer, J., Gardell, S. J. *J. Biol.Chem*. **2001**, *267*, 10366-10373
31. Haniu M, Denis P, Young Y, Mendiaz EA, Fuller J, Hui JO, Bennett BD, Kahn S, Ross S, Burgess T, Katta V, Rogers G, Vassar R, Citron M (2000) *J Biol Chem*. **276,** 10879-87
32. Benjannet S, Elagoz A, Wickham L, Mamarbachi M, Munzer JS, Basak A, Lazure C, Cromlish JA, Sisodia S, Checler F, Chretien M, Seidah NG. (2001) *J Biol Chem.* **276**, 10879-87
33. Hussain I, Powell D, Howlett DR, Tew DG, Meek TD, Chapman C, Gloger IS, Murphy KE, Southan CD, Ryan DM, Smith TS, Simmons DL, Walsh FS, Dingwall C, Christie G (1999) *Mol Cell Neuroscience* **14**, 419-427
34. Gruninger-Leitch F, Schlatter D, Kung E, Nelbock P, Dobeli H. *J Biol Chem*. (2002) **277**, 4687-93
35. Roberds SL, Anderson J, Basi G, Bienkowski MJ, Branstetter DG, Chen KS, Freedman SB, Frigon NL, Games D, Hu K, Johnson-Wood K, Kappenman KE, Kawabe TT, Kola I, Kuehn R, Lee M, Liu W, Motter R, Nichols NF, Power M, Robertson DW, Schenk D, Schoor M, Shopp GM, Shuck ME, Sinha S, Svensson KA, Tatsuno G, Tintrup H, Wijsman J, Wright S, McConlogue L. (2001) *Hum Mol Genet.* **10**, 1317-24
36. He X, Li F, Chang WP, Tang J. (2005) *J Biol Chem.* **280**, 11696-703
37. Huse JT, Pijak DS, Leslie GJ, Lee VM, Doms RW. (2000) *J Biol Chem.* **275,** 33729-37

38. Capell A, Meyn L, Fluhrer R, Teplow DB, Walter J, Haass C. (2002) *J Biol Chem.* **277,** 5637-43
39. Lichtenthaler SF, Dominguez DI, Westmeyer GG, Reiss K, Haass C, Saftig P, De Strooper B, and Seed B (2003) *J Biol Chemistry* **278**, 48713-9
40. Kitazume, S., Tachida, Y., Oka, R., Shirotani, K., Saido, T. C., and Hashimoto, Y. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 13554–13559
41. Li, Q., and Sudhof, T. C. (2004) *J. Biol. Chem*. **279**, 10542–10550
42. von Arnim CA, Kinoshita A, Peltan ID, Tangredi MM, Herl L, Lee BM, Spoelgen R, Hshieh TT, Ranganathan S, Battey FD, Liu CX, Bacskai BJ, Sever S, Irizarry MC, Strickland DK, Hyman BT (2005) *J Biol Chem.* **280**, 17777-85
43. Russo T, Faraonio R, Minopoli G, De Candia P, De Renzis S, Zambrano N (1998) *FEBS Letters* **434**, 1-7
44. He X, Chang WP, Koelsch G, and Tang J (2002) *FEBS Letters* **524**, 183-187
45. Gao Y, Pimplikar SW (2001) *Proc Natl Acad Sci USA* **98,** 14979-84
46. He, X., Zhu, G., Koelsch, G., Rodgers, K. K., Zhang, X. C., and Tang, J. (2003) *Biochemistry* 42, 12174–12180
47. Simons K, Ikonen E(1997) *Nature*, **387**, 569-572
48. S.J. Singer and G.L. Nicolson (1972), *Science* **175,** 720–731
49. Edidin M (2003) *Annu. Rev. Biophys. Biomol. Struct.* **32**, 257–8
50. Engelman (2005) *Nature*
51. Ehehalt R, Keller P, Haass C, Thiele C, and Simons K (2003) *J Cell Biol* **160**, 113-123
52. Bodovitz S, Klein WL (1996) *J Biol Chem* **271**, 4436-4440
53. Puglielli L, Ellis BC, Saunders AJ, Kovacs DM (2003) *J Biol Chem* **278**, 19777-19783
54. Cordy JM., Hussain I., Dingwall C, Hooper NM., and Turner AJ (2003) *Proc Natl Acad Sci USA* **100**, 11735-11740
55. Rouvinski A, Gahali-Sass I, Stav I, Metzer E, Atlan H, Taraboulos A (2003) *Biochem Biophys Res Commun.* **308**, 750-758
56. Bouillot C, Prochiantz A, Rougon G, and Allinquant B (1996) *J Biol Chem* **271**, 7640–7644
57. Riddell DR, Christie G, Hussain I, and Dingwall C (2001) *Curr Biol* **11**, 1288–1293
58. Simons M, Keller P, De Strooper B, Beyreuther K, Dotti CG, and Simons K (1998) *Proc Natl Acad Sci USA* **95**, 6460–6464
59. Lang T, Bruns D, Wenzel D, Riedel D, Holroyd P, Thiele C, Jahn R (2001) *EMBO J.* **20**, 2202-22013
60. Rodal SK, Skretting G, Garred Ø, Vilhardt F, van Deurs B, Sandvig K. (1999) *Mol. Biol. Cell* **10**, 961-974
61. Subtil A, Gaidarov I, Kobylarz K, Lampson MA, Keen JH, McGraw TE. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 6775-6780
62. Pike LJ, Miller JM. (1998). *J Biol Chem* **273**, 22298-22304
63. Yates AJ, Rampersaud A. (1998) Ann N Y Acad Sci **845**, 57-71
64. Meuillet EJ, Mania-Farnell B, George D, Inokuchi JI, Bremer EG. (2000) *Exp Cell Research* 256, 74-82
65. Bogdanov M, Dowhan W. (1998) *EMBO J.* **17**, 5255-64

66. Cornea RL, Thomas DD (1994) *Biochemistry* 33, 2912-20
67. Arsov Z, Schara M, Zorko M, and Strancar J (2004) *Eur Biophys J* **33,** 715–725
68. Gopalakrishnan M, Forsten-Williams K, Nugent MA, and Täubery UC (2005) *Biophys J* **89,** 3686–3700
69. Nicolau DV Jr, Burrage K, Parton RG, Hancock JF. (2006) *Mol Cell Biol.* **26**, 313-23
70. Rigaud JL (2002) *Braz J Med Biol Res*, **35**, 753-766
71. Kahya N, Brown DA, and Schwille P. (2005) *Biochemistry* **20**, 7479-7489
72. Fujiki Y, Hubbard AL, Fowler S, and Lazarow PB (1982) *J Cell Biol*, **93**, 97–102
73. Magde D, Elson E, and Webb WW (1972) *Phys. Rev. Lett.* **29,** 705-708
74. Schägger H, von Jagow G (1991) *Anal Biochem* **199**, 223-231
75. Bogdanov M, Sun J, Kaback HR, Dowhan W (1996) *J Biol Chem* **271**, 11615-11618
76. van der Brink-van der Laan E, Killian JA, and de Kruijff B (2004) *Biochim Biophys Acta* **1666**, 275-288
77. Riddell DR, Christie G, Hussain I, and Dingwall C (2001) *Curr Biol* **11**, 1288–1293
78. Zha Q, Ruan Y, Hartmann T, Beyreuther K, Zhang D. (2004) *Mol Psychiatry*, **9**, 946-952
79. Kakio A, Nishimoto S, Yanagisawa K, Kozutsumi Y, Matsuzaki K (2002) *Biochemistry*, **41**, 7385-7390
80. Westmeyer GG, Willem M, Lichtenthaler SF, Lurman G, Multhaup G, Assfalg-Machleidt I, Reiss K, Saftig P, Haass C. (2004) *J Biol Chem* **279**, 53205-12
81. Veenhoff LM, Heuberger EHML, and Poolman B (2002) *TRENDS Biochem Sci* **27**, 242
82. Newton AC (1993) *Annu Rev Biomol Biophys Struct* **22**, 1-25
83. Poveda JA, Encinar JA, Fernandez AM, Mateo CR, Ferragut JA, Gonzalez-Ros JM. (2002) *Biochemistry* **41**, 12253-12262
84. Buckland AG and Wilton DC, (2000) *Biochim Biophys Acta* **1483**, 199-216
85. Denkins YM, Schroit AJ (1986) *Biochim Biophys Acta* **862**, 343-351
86. Pautot S., Frisken BJ, and Weitz DA (2003) *Proc Natl Acad Sci USA* **100**, 10718-10721
87. McLaughlin (1977) *Curr Top Membr Transport* **9**, 71-144
88. de Almeida RF, Fedorov A, Prieto M (2003) *Biophys J* **85**, 2406-16
89. Kahya N, Brown DA, and Schwille P. (2005) *Biochemistry* **20**, 7479-7489
90. de Almeida RFM, Loura LMS, Fedorov A, and Prieto M (2005) *J. Mol. Biol.* **346**, 1109–1120
91. Meder D, Moreno MJ, Verkade P, Vaz WL, Simons K. (2006) *Proc Natl Acad Sci U S A* **103**, 329-34
92. Berry H (2002) *Biophys J* **83**, 1891-1901
93. Veatch SL, Keller SL (2003) *Biophys J* **85,** 3074–3083
94. Kahya N, Pecheur E-I, de Boeij WP, Wiersma DA, and Hoekstra D (2001) *Biophys J* **81**, 1464–1474

95. Kahya N, Scherfeld D, Bacia K, Poolman B, and Schwille P (2003) *J Biol Chem* **278,** 28109

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8. Declaration

I herewith declare that I have produced this thesis without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This thesis has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from October 2002 to May 2006 under the supervision of Prof. Dr. Kai Simons at the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden.

Dresden, May 9, 2006